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EVALUATION OF IMMERSION IMMUNISATION OF ATLANTIC SALMON (*SALMO SALAR*) AGAINST YERSINIOSIS

Thu Diem Nguyen

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University of Tasmania

Launceston, Tasmania

APPROVALS

Doctor of Philosophy Dissertation

Immersion immunisation of Atlantic salmon against Yersiniosis

By Thu Diem Nguyen

BSc, MAppSc (Aquaculture)

Supervisor: _____

Professor Barbara F. Nowak

DECLARATIONS BY THE AUTHOR

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The research associated with this thesis abides by the international and Australian codes on human and animal experimentation, the guidelines by the Australian Government's Office of the Gene Technology Regulator and the rulings of the Safety, Ethics and Institutional Biosafety Committees of the University (AEC approval numbers: A0012285).

Signed: _____ Dated: 10 July 2015

(Thu Diem Nguyen)

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ABBREVIATIONS

ANOVA	analysis of variance
AMPs	antimicrobial peptides
AU	arbitrary unit
BCP	bromochloropropane
BSA	bovine serum albumin
cDNA	complementary deoxyribonucleic acid
CFU	colony forming units
CTLs	cytotoxic T lymphocytes
d	day
DNA	deoxyribonucleic acid
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
EF1a	elongation factor 1a
ELISA	enzyme-linked immunosorbent assay
ERM	enteric redmouth disease
ESC	enteric septicaemia of catfish
ETOH	ethanol
g	gravity
g	gram
GALT	gut-associated lymphoid tissue
GIALT	gill-associated lymphoid tissue
h	hour
HRPO	horseradish peroxidase
IFAT	immunofluorescence antibody technique
IFN	interferon
IgA	immunoglobulin A
IgD	immunoglobulin D
IgE	immunoglobulin E
IgG	immunoglobulin G
IgM	immunoglobulin M
IgT	immunoglobulin T
ILUs	inter-lamellar units
i.p.	intraperitoneal
IRFs	interferon regulatory factors
kg	kilogram
kHz	kilohertz
L	litre
LB	Luria Bertani media

LPS	lipopolysaccharide
M	mole
MALT	mucosa associated lymphoid tissue
mg	milligram
MHC	major histocompatibility complex
MHz	megahertz
min	minute
mL	millilitre
mm	millimetre
mM	micromole
MMC	Melano-macrophage center
mRNA	messenger ribonucleic acid
n	number (of individuals/samples)
NETs	neutrophil extracellular traps
NK	natural killer
NLRs	NOD-like receptors
nM	nanomole
nm	nanometre
OD	optical density
PAMP	pathogen associated molecular pattern
PBS	phosphate buffered saline PBS
PCR	polymerase chain reaction
PRR	pattern recognition receptor
qPCR	quantitative real-time RT-PCR
RLRs	RIG-I-like receptors
RNA	ribonucleic acid
ROS	reactive oxygen species
rpm	revolutions per minute
RPS	relative percent survival
RT	room temperature
RT-PCR	reverse-transcriptase polymerase chain reaction
s	second
SALT	skin-associated lymphoid tissue
SD	standard deviation
SDS	sodium dodecyl sulphate
S.E.	standard error
SPSS	statistical package for the social sciences
TBE	tris borate EDTA
TBS	tris-buffered saline
TCR	T-cell receptors

T_H	T helper
TLRs	toll- like receptors
TMB	3,3´,5,5´-tetramethyl benzidine
TSA	tryptone soy agar
UV	ultraviolet
V	volts
W	watts
X	times
µg	microgram
µm	micrometre
µL	microliter

NOTE REGARDING THESIS STRUCTURE

The first chapter of this thesis is written as a general introduction and review of relevant literature that relate to the experiments of this thesis. The experiment in Chapter 4 was combined with another concurrently enrolled PhD candidate but Chapter 4 in this thesis presents only the results obtained from experimental analysis performed by the candidate to meet the rules governing doctoral candidature in Australia, namely no material can be included into more than one thesis. Therefore the combined results which are currently prepared for submission to a journal are presented in Appendix 1. The reference style of Aquaculture journal has been adopted for this thesis, and a list of references of all chapters in this thesis is included at the end of the thesis.

EXECUTIVE SUMMARY

Yersinia ruckeri, a Gram-negative bacteria, is a pathogen which causes yersiniosis and significant losses in farmed Atlantic salmon (*Salmo salar*) in the Southern Hemisphere. Currently, Yersinivac-B, prepared from formalin killed whole-cells, is a commercial bacterin-based vaccine manufactured by MSD Animal Health. It is delivered by bath immersion vaccination against *Y. ruckeri* for most Tasmanian Atlantic salmon. Significant mortality events may still occur despite vaccination.

This thesis focused on the improvement of yersiniosis vaccine efficacy for Atlantic salmon by using different administrated methods of immersion immunisation. The following aims were addressed in this thesis:

- Evaluate the effects of different inactivation methods of *Y. ruckeri* on the efficacy of single dip vaccines including formalin inactivation, ammonium sulphate inactivation, and pH-lysed then formalin treated inactivation.

- Compare hyperosmotic pretreatment with direct immersion in Atlantic salmon (*Salmo salar*) by using an ammonium sulphate inactivated *Y. ruckeri* whole-cell vaccine.

- Investigate the vaccine performance of immersion vaccination with single dip and double dip vaccination of small fish at hatchery stage.

In this thesis, three different experiments were carried out to obtain the results. The first experiment where the vaccine efficacy of Atlantic salmon was determined using the relative percent survival (RPS), antibody production levels, and carrier status of *Y. ruckeri* and gene expression. The results showed that ammonium sulphate was successfully used for *Y. ruckeri* inactivation and demonstrated further potential for bacteria inactivation using this method for commercial fish vaccines. Additionally, the serum humoral antibody levels did not correlate with the RPS and were not a good marker of protection of the fish against *Y. ruckeri* infection. The prevalence of asymptomatic

carriers was low. The gene expression was consistent with previous successful use as a biomarker of vaccine success in Atlantic salmon against *Y. ruckeri*.

In the second experiment, the vaccine efficacy was assessed when ammonium sulphate inactivated bacterin was combined with hyperosmotic infiltration in vaccination. The results showed that ammonium sulphate vaccinated fish with hyperosmotic infiltration had significantly better survival than the unvaccinated fish with 25.6% higher in the RPS. It has demonstrated that hyperosmotic infiltration could improve protection of a vaccine against *Y. ruckeri* for Atlantic salmon and has the potential to be used with other bacterin-based immersion vaccines.

The vaccine efficacy using single dip and double dip immersion vaccination at first feeding was tested to determine potential of protection against the early outbreaks of yersiniosis in the hatchery stage. There was no significant difference in the survival of salmon regardless of vaccination. In addition, expression levels of IgM gene were not different between the unvaccinated fish (control) and other two groups of vaccinated fish. This suggested that the vaccine did not work with the small fry that were vaccinated twice at 0.13 g and 1 g (2 dips) and single vaccinated at 1 g size.

This thesis provided further understanding into different methods of immersion immunisation to yersiniosis in Atlantic salmon. Ammonium sulphate was successfully used for *Y. ruckeri* inactivation in vaccine production. The minimum fish size for the earliest vaccination by immersion needs to be investigated further.

CHAPTER 1.

GENERAL INTRODUCTION

1.1. IMMUNE RESPONSE OF TELEOSTS

The immune system of fish is similar to higher vertebrates and protects fish from threats such as bacteria, parasites and viruses (Lieschke & Trede, 2009; Uribe et al., 2011). The thymus, kidney and spleen are considered the primary lymphoid organs in teleosts (Zapata et al., 2006). The thymus is located near the gill cavity and is responsible for the production and proliferation of T-cells (Koppang et al., 2010). The kidney is the largest site of haematopoiesis and is the equivalent of the bone marrow in vertebrates (Zapata et al., 2006). The spleen is involved in haematopoiesis, represents the major peripheral lymphoid organ in jawed vertebrates showing erythropoiesis and thrombopoiesis activity (Rombout et al., 2005) and is involved in antigen capture (Press & Evensen, 1999). Mucosa associated lymphoid tissue (MALT) includes gut-associated lymphoid tissue (GALT), skin-associated lymphoid tissue (SALT), and the more recently described gill-associated lymphoid tissue (GIALT) (Salinas et al., 2011). The GALT includes the lamina propia and the intraepithelial compartments, is scattered along the intestine.

The immune response can be divided into innate immunity or non-specific immune response, and adaptive immunity or specific immune response. Innate immunity performs an important role for survival from their early embryonic stages of life (Uribe et al., 2011). It is present in both vertebrates and invertebrates, is promptly available to combat infections and is the first line of defence against pathogens (Jørgensen, 2014). Innate immunity can be divided into a sensing arm and an effector arm. The sensing arm deals with how fish recognise pathogens while the effector arm deals with coordination of cellular responses to combat infection. Immune response includes cellular and humoral components and both are present in the sensing arm and the effector arm (Jørgensen, 2014).

Myeloid cells, professional phagocytes that engulf and kill pathogens, include mononuclear and polymorphonuclear phagocytes, and are part of the innate immune response in vertebrates. The mononuclear phagocytes are efficient at presenting antigens to T-cells of the adaptive immune system (Jørgensen, 2014). Neutrophils are the main type of

polymorphonuclear phagocytes in fish (Hine, 1992). In fish, both macrophages and neutrophils are key inflammatory cells and are mobilized upon tissue injury or infection (Jørgensen, 2014). Melanomacrophages contain pigments including melanin. These cells can be found in the spleen, head kidney, as well as inflammations sites. They are assumed to be a part of the antigen-presenting cell system (Agius & Roberts, 2003).

Phagocytes have two main tasks: phagocytosis and intracellular killing of pathogenic microorganisms. The pathogens are trapped into phagosomes or membrane-delimited compartments which then fuse with granules to form a phagolysosome. Within this phagolysosome, the pathogen is digested or effectively killed by the enzymes, antimicrobial peptides (AMPs) and reactive oxygen species (ROS) (Jørgensen, 2014). In addition, neutrophils can eliminate pathogenic microorganisms by the release of neutrophil extracellular traps (NETs) or antimicrobial proteins and extracellular structures composed of DNA (Brinkmann & Zychlinsky, 2007). The mononuclear fish phagocytes can express MHC class II and have the molecular machinery essential for antigen processing and presentation (Iliev et al., 2010; Wittamer et al., 2011). Natural killer (NK) cells are cytotoxic lymphocytes that can recognise infected and stressed cells and provide rapid responses to kill these cells by releasing inflammatory cytokines. The role of NK cells is similar to cytotoxic T-lymphocytes of adaptive immune responses in vertebrate but NK cells have the ability to recognise infected cells without need of major histocompatibility complex (MHC) and prior sensitisation (Jørgensen, 2014).

Unlike the adaptive immune response, the innate immune response is available immediately to fight pathogenic agents and it responds to pathogen-associated molecular patterns (PAMPs). These PAMPs are detected by the soluble and cell-associated germline-encoded pathogen recognition receptors (PRRs) (Jørgensen, 2014). The innate immune receptors that recognise bacteria and virus in fish have been studied and some PRRs have been identified in fish including the toll-like receptors (TLRs), C-type lectins, RIG-I-like receptors, complement components and NOD-like receptors (Zhu et al., 2013).

Toll-like receptors (TLRs) represent one of the best studied PRRs with at least 16 types being reported in different fish species (Palti, 2011). Two primary subtypes of TLRs are identified in humans including type I (TLR1, 2, 4, 5, 6 and 10) which identify microbial lipids and sugars derived from different fungi and bacteria, while type II (TLR3, 7, 8 and 9) mainly respond to nucleic acids derived from bacteria and virus. In fish, representatives from both of these subtypes are found evenly with greater diversity of six more non-mammalian TLRs including TLR14 (similar TLR1 and 2), TLR19, 29, 21, 22 and 23 (Oshiumi et al., 2003; Xiang et al., 2010). TLR5, a membrane-bound receptor in mammals, recognises bacterial flagella through their flagella component and it has been reported from rainbow trout (*Onchorhynchus mikiss)* (see Tsujita et al., 2004) and Atlantic salmon (*Salmo salar*) (see Tsoi et al., 2006).

The NOD-like receptors (NLRs) and the RIG-I-like receptors (RLRs), two additional families of innate receptors, mainly related in bacterial or viral defence, have been found in fish (Laing et al., 2008). NOD1 and NOD2 are the best known of the NLR family members and these NLRs which are involved in inflammation, antibacterial and antiviral defences are present in fish in fish (Chang et al., 2011a; Sha et al., 2009). RIG-I, MDA-5 and LGP2 are three members of the RLRs. These RLRs are involved in viral infection and has been found in rainbow trout, channel catfish (*Ictalurus punctatus*) and grass carp (*Ctenopharyngodon idella*) (see Chang et al., 2011b; Rajendran et al., 2012; Wang et al., 2012).

Lectins, a group of sugar binding proteins, are important components of the innate immune system. They bind to carbohydrate structures from bacteria, virus, fungi and animals. In fish, diverse families of lectins recognise and agglutinate bacteria, virus, parasites and fungi. They also participate in downstream effector functions, such as agglutination and opsonisation of enteric bacteria (Vasta et al., 2011). C-type lectins (CTLs), members of the PRR family, have been identified in several fish species such as rainbow trout, zebrafish, common carp and turbot (Vasta et al., 2011).

Most TLRs, NLRs and RLRs, not only bind to their PAMPs, but also lead to activation of NFkB and interferon regulatory factors (IRFs) (Kumar et al., 2011). NFkB activation

results in induction of DC maturation (CD80, CD83, CD86), enhanced inflammatory cytokine responses, chemokines and chemokine receptors (Janeway & Medzhitov, 2002). IRFs activation mainly results in direct antiviral activity by interferon (IFN) induction, and also contributes to the host immune response (Kumar et al., 2011).

Cytokines, small cell-signalling protein molecules, include interleukins and IFNs as immunomodulating agents (Jørgensen, 2014). About 35 interleukins are presently described in mammals and many direct homologues of these molecules are present in bony fish (Secombes et al., 2011). The cytokines IL-1β, TNF-α and type IFNs are the best characterised fish cytokines, and are crucial cytokines secreted from innate immune cells (Jørgensen, 2014). The IL-1 family has a key role in the inflammatory responses and mammalian IL-1α, IL-1β and IL-18 have been broadly characterised among their 11 members (Barksby et al., 2007). In fish, only homologs to IL-1β and IL-18 have been identified, and IL-1β genes have been cloned from salmon (Ingerslev et al., 2006), trout (Zou et al., 1999), carp (Engelsma et al., 2003) and cod (Seppola et al., 2008). Salmonids have three different IL-1β genes (IL-1β1, 2 and 3) and expression of IL-1β has been found in the spleen, head kidney and gills (Ingerslev et al., 2006).

A member of TNF family is TNF-α which is involved in cellular signalling pathways in teleost (Wiens & Glenney, 2011). TNF-α is a type II transmembrane glycoprotein that contains an extracellular C-terminal domain and a cytoplasmic tail. It is constitutively expressed in the gills and head kidney of rainbow trout and produced by activating macrophages and T lymphocytes (Laing et al., 2001). Important defence, produced during viral infections are IFNs which interfere with viral replication. Viral dsRNA or ssRNA act as PAMPs that are identified by PRRs and induce activation of transcription factors, followed by expression of type I IFNs which creates an antiviral state in the infected cell and alerts immune cells (Jørgensen, 2014). Atlantic salmon has at least 11 IFN-genes with three subtypes (Sun et al., 2009). IFN has a direct activity against pathogenic viruses by inducing the expression of IFN-stimulates genes (ISGs) such as Mx, ISG15 and PKR (Caipang et al., 2003; Larsen et al., 2004; Zhu et al., 2008).

The complement system, playing an important role in innate immunity, is made up of about 30 distinct plasma proteins and membrane-associated proteins, has ability to fight infections by opsonising pathogens and inducing a series of inflammatory responses (Janeway et al., 2001; Jørgensen, 2014). There are three different pathways of complement activation on pathogen surfaces including classical, alternative and lectin pathway. The classical pathway is commenced by the binding of C1b protein as a recognition unit that mainly recognises antibodies in the immune complexes. The lectin pathway is activated upon binding of the mannose-binding lectin (MBL) that is a serum protein, to sugar residual on the surface of the invading microorganism. The alternative pathway can be initiated by spontaneous activation of C3 binds to the surface of pathogen. The generation of C3 convertase, cleaves C3 to generate C3a and C3b, is the main function of all three pathways. C3b binds covalently to the invading pathogen and this pathogen was destroyed by phagocytes (Jørgensen, 2014).

The adaptive immune system is triggered by some cells of the innate immune response such as macrophages and dendritic cells, when some pathogens still invade and establish infections (Mutoloki et al., 2014). This response has evolved early in vertebrate evolution, about 450 million years ago and it is found in all jawed vertebrates. The adaptive response relies upon antigen recognition and has exquisite specificity. It takes several days to weeks to develop but it improves and responds more effectively upon subsequent encounters as the same pathogen due to the ability to remember the invaders (Mutoloki et al., 2014). Improving the knowledge of the adaptive immune responses of fish is important for vaccine development (Warr, 1996).

The lymphocytes, the key cell type of the adaptive immunity, including the B-cell and T-cells, are responsible for specificity of antigen recognition and initiation of the adaptive immune response (Magnadottir, 2010). Lymphocytes initiate from the bone marrow in mammals, have a lymphoid lineage that is different from the innate system as a myeloid lineage (Mutoloki et al., 2014). The humoral response involves B-cells while the cell-mediated response associates with T-cells (Magnadottir, 2010). In fish, both B-cells and T-cells originate from the head kidney as there is no bone marrow (Mutoloki

et al., 2014). The head kidney is important for the maturation of B-cells, while the maturation of T lymphocytes occurs within the thymus (Salinas et al., 2011). In teleost fish, the head kidney and spleen are the main antigen trapping organs (Press & Evensen, 1999). Melano-macrophage centers (MMC) in head kidney and spleen are structures of antigen trapping and primitive analogs of the germinal centers of higher vertebrates (Mutoloki et al., 2014).

Immunoglobulins, important humoral components of the adaptive immune system, have been found in teleosts and include three classes. IgM is the primary antibody and is mainly produced by plasma cells and plasmablasts located in the head kidney (Bromage et al., 2004). This antibody isotype plays an important role as a marker for protection against bacterial and viral disease in fish (Mutoloki et al., 2014). The second isotype, IgD has been detected at the gene level in a number of fish species (Laurent & Perry, 1990; Silverstone & Hammell, 2002), but only detected in channel catfish (*Ictalurus punctatus*) at the protein level as a monomer and lacking the variable region (Edholm et al., 2010). IgD-armed granulocytes were present. The third isotype, IgT is predominantly found in the gut mucosa of several fish species. Pathogens affecting the gut induce IgT expression (Zhang et al., 2011). IgT is found in the mucus of the trout skin and in the gut mucosa (Sunyer, 2012) as well as gills (Chettri et al., 2012; Stevenson, 1988), suggesting that IgT is important in mucosal immunity. However, the mechanisms of protection from this antibody remain poorly understood with few studies being conducted on fish (Salinas et al., 2011).

T lymphocytes or T cells play a central role in cell-mediated immune responses (Mutoloki et al., 2014). Depending on their T-cell receptors (TCR), T cells can be divided into αβ or γδ T-cells. The common traits like TCR, CD3, CD28 and CD45 are shared with mature T lymphocytes and these traits are involved in pathogen recognition and signalling or are on the surface molecules. Only antigens that are associated with MHC molecules or present on surfaces of other cells are recognised by T-cells. Depending on the function of cytotoxic or helper, T-cells are further divided into two groups. Cytotoxic T lymphocytes (CTLs), the effector cells of the cytotoxic response, are

involved in direct killing of abnormal or infected cells by secreting molecular to induce apoptosis. The target cells which display abnormal proteins or fragments of pathogens in association with the MHC-I molecules on their surface are identified by CTLs. CD3 chains, CD28 and other T-cell co-inhibitory and co-stimulatory molecules were described in several species (Boardman et al., 2012; Maisey et al., 2011; Øvergård et al., 2009; Typical, 2006). However, the functional aspects of putative responses of different T-cell subsets still remains poorly understood. CTLs express CD8 molecules and TCR co-receptors which are involved in the MHC-I interaction of the cells. On the other hand, T helper (T_H) cells act directly by secreting cytokines that regulate in the active immune response to a foreign threat (Mutoloki et al., 2014). A surface glycoprotein CD4 is expressed by T_H cells, involved in the cellular interaction with MHC-II proteins. CD4 and CD4-like genes have been reported in many teleost fish (Dijkstra et al., 2006; Moore et al., 2009). Depending on the type of cytokine profile producing, T_H cells are categorised into several regulator and effector subsets. T_H1 cells, important in cell-mediated responses, have been found in many fish species as IFN-γ (Zou & Secombes, 2011). T_H2 cells relate in humoral response, have not been functionally described in fish. T_H17 cells, enhance responses to extracellular bacteria, secrete cytokines IL-17A to F in mammals. Five form of IL-17 has been found in zebrafish (Gunimaladevi et al., 2006). T_H17 cells also secrete IL-22 that was found in teleost fish. Another T-cell subset, T_H22 secretes IL-22 but it is incapable of producing IL-17 (Eyerich et al., 2010).

1.2. FISH VACCINATION

Fish vaccines are an effective way to control fish diseases and increase fish production in aquaculture (Plant & LaPatra, 2011). More than 17 fish species have been vaccinated against more than six viral diseases and 22 different bacterial diseases in more than 40 countries (Brudeseth et al., 2013). Vaccines have been used in salmon farming for approximately 30 years and the use of vaccines has increased salmon production. The use of oil-adjuvanted vaccines for controlling salmon disease reduced the use of antibiotics on Norwegian fish farms from 50,000 kg of antibiotics in 1987 to less than 2,000 kg by 1997, while production increased from 50,000 tonnes to 350,000 tonnes

(Pridgeon et al., 2010; Sommerset et al., 2005). In other parts of the world, more than 900 million fish have been vaccinated against enteric septicaemia of catfish (ESC) since 2002 and this has given 13% higher fish survival in fish production (Pridgeon et al., 2010).

Fish vaccines can be administered by injection, immersion or orally. In general, intraperitoneal injection (i.p.) vaccination has the greatest efficacy and requires less vaccine, but can only be applied to larger juveniles and adult fish (more than 20 g fish). Anderson and Nelson (1974) showed that i.p. injection of a bacterin provided the most effective protection in terms of duration and relative percent survival (RPS) values. However, labour costs associated with i.p. vaccination are high and the vaccination can result in stress for the fish (Ototake et al., 1999). Furthermore, side effects associated with the injection site such as peritoneal lesions are of increased concern when oil based adjuvants are administered with i.p. vaccination (Skirtun et al., 2013).

Immersion vaccination is a natural route of antigen entry that can overcome the disadvantages of delivery by injection (Moore et al., 1998). However, it often gives shorter duration of protection and needs a significantly greater volume of vaccine (Dixon & Becker, 2011). Dip and bath are two application methods of immersion vaccination (Komar et al., 2006). In dip vaccination, fish are immersed in a high concentration of vaccine for a very short time, normally 30 seconds to a few minutes. In bath vaccination, fish are immersed for a longer period of exposure (one to several hours) (Ross et al., 1966) in a low vaccine concentration.

Fish vaccines against bacterial diseases can be made up of chemically or heat-inactivated whole cells, inactivated soluble cell extracts, cell lysates, attenuated live cells, purified subcellular components, serum (for passive immunization) and mixtures of the components (Austin, 1984). However, the use of inactivated whole cells is the simplest and most common approach to producing vaccines for fish. Several methods for inactivating bacterial cells have been used and include the treatment with chemicals, such as chloroform, formalin, phenol, heat, sonication, and lysis with sodium dodecyl

sulphate (SDS) or with sodium hydroxide at pH 9.5 have tested for experimental fish vaccines (Austin, 1984). Formalin inactivation of cells is most commonly used for commercial fish vaccines (Austin, 1984; Sommerset et al., 2005). However, a few studies have compared different methods of inactivation. Amend et al. (1983) showed that chloroform inactivation of *Y. ruckeri* bacterins did not provide any advantage over formalin inactivation. The procedure of heat or formalin inactivation of bacterins may produce significant alterations of the antigens (Laurent & Hebibi, 1989; Roberts & Powell, 2003). *Aeromonas salmonicida* has been inactivated by chloroform, formalin, disruption with SDS, sonication and heat-killing for incorporation in fish vaccines and the results showed that formalin- inactivated cells of *A. salmonicida* fared better than their sonication and disruption with SDS counterparts (Antipa & Amend, 1977; Franklin, 1990; Katoh & Kaneko, 2003; Pritchard, 2003).

Several studies have demonstrated ways to improve or enhance antigen uptake or vaccine efficacy in fish (Table 1.1). For example the use of ultrasound, considered as a new method of administrating vaccine to fish, has been shown to increase survival rates and antibody titres (Table 1.1, Navot et al., 2004; Navot et al., 2011; Zhou et al., 2002a; Zhou et al., 2002b). This method was as effective as intraperitoneal injection and required less volume of vaccine than bath immersion. In addition, hyperosmotic infiltration, which immerses the fish in 4.5% to 5.3% sodium chloride for a short period of time before vaccine administration, can enhance antigen or vaccine uptake (Table 1.1, Huising et al., 2003; Ototake & Nakanishi, 1992; Ototake et al., 1992). Furthermore, prolonged exposure to antigens during immersion vaccination can also increase particular uptake or vaccine effectiveness (Moore et al., 1998; Ototake et al., 1998; Ototake et al., 1999). Some immunostimulants such as trypsin, levamisole, propiscin and adjuvant are also used to elevate vaccine efficacy (Table 1.1).

Table 1.1 Methods to improved antigen uptake or vaccine efficacy in fish

No	Treatment	Vaccine/Antigen	Species (size)	Other conditions (t^o, exposure)	Effect	References
1	Ultrasound	BSA	Goldfish (*Carassius auratus*) 20-30 g	25 ± 1 ^oC Ultrasound: 1 MHz, 1 min to 3 MHz, 10 min; 2 or 0.4% BSA solution – 10 min	↑ antigen transport through the skin ↑ antibody production ↓ antigen concentrations	Navot et al. (2004)
2	Ultrasound	*Vibrio alginolyticus*	Grouper (*Epinephalus awoara*) 20.5-22.5 g	Salinity 33‰ 28-30 ^oC Ultrasound: 35 kHz (frequency) & 175 mW/cm^2 (intensity), 2-4 min	↑ antibody titres ↑ survival rates Ultrasonic vaccination = intraperitoneal injection	Zhou et al. (2002b)
3	Ultrasonic immunization	Mixed vaccine against *Vibrio alginolyticus* & *V. anguillarum*	Sea bream (*Pagrus major*) 41.6 ± 5.2 g	24-28 ^oC Ultrasound: 35 kHz (frequency) & 280 mW/cm^2 (intensity), 3-6 min	↑ antibody titres ↑ survival rates Ultrasonic vaccination = intraperitoneal injection Reused the vaccine up to five times	Zhou et al. (2002a)
4	Ultrasound	*Aeromonas salmonicida*	Goldfish (*Carassius auratus*) 20 g	25 ± 1 ^oC Ultrasound: 1 MHz frequency, 1 min	↑ morbidity & cumulative protection ↓ Petechiae ↓ Ulcers	Navot et al. (2011)
5	Prolonged exposure period on soluble antigen uptake	BSA	Rainbow trout (*Oncorhynchus mykiss*) 15.0 ± 3.4 g	15 ^oC 3 min – 48 hours	↑ plasma BSA concentration	Ototake et al. (1998)

6	Prolonged exposure	BSA-conjugated 1 µL fluorescent latex microspheres	Rainbow trout (*Oncorhynchus mykiss*) 1.5 ± 2.99 g	15 °C 3 min – 48 hours	↑ particular uptake	Moore et al. (1998)
7	Prolonged exposure	*Vibrio ordalii*	Rainbow trout (*Oncorhynchus mykiss*) 1.2 ± 0.5 g	15 °C 3 min – 24 hours	↑ vaccine effectiveness	Ototake et al. (1999)
8	Hyperosmotic infiltration	Bovine serum albumin (BSA)	- Rainbow trout (*Oncorhynchus mykiss*) 18 ± 3 g - Yellowtail (*Seriola quinqueradiata*) 40 ± 8 g - Chum salmon (*Oncorhynchus keta*) 15-17 g - Tilapia (*Oreochromis aureus*) 156-157 g	15 °C 20 °C 15 °C 25 °C 5.3% NaCl - 3 min	↑ concentration of plasma BSA (freshwater -acclimatized fish)	Ototake et al. (1992)
9	Hyperosmotic	*Aeromonas salmonicida* bacterin, *A. salmonicida* lipo-polysacchride (LPS), Bovine serum albumin (BSA)	Common carp (*Cyprinus carpio*)	23 °C 4.5% NaCl - 2 min → vaccine solution 10 min	↑ vaccine uptake ↑ vaccine efficacy	Huising et al. (2003)
10	Hyperosmotic infiltration	Bovine serum albumin (BSA)	- Rainbow trout (*Oncorhynchus mykiss*) 18 ± 3 g	15 °C	BSA levels of seawater-acclimatized fish < freshwater -acclimatized fish	Ototake and Nakanishi (1992)

			- Yellowtail (*Seriola quinqueradiata*) 40 ± 8 g - Chum salmon (*Oncorhynchus keta*) 15-17 g - Tilapia (*Oreochromis aureus*) 156-157 g	20 ºC 15 ºC 25 ºC 5.3% NaCl - 3 min		
11	Immunostimu-lants (QAC-quaternary ammonium; ISK-short-chain polypeptide; Levamisole)	*Aeromonas salmonicida* O antigen	- Rainbow trout (*Oncorhynchus mykiss*) 20-30 g	11 ± 2 ºC Immunostimulant 30 min → antigen 2 min	↑ neutrophil oxidative activity ↑phagocytic uptake ↑ protection levels	Jeney and Anderson (1993)
12	Combined immer-sion/puncture immunization	*Streptococcus iniae*	Rainbow trout (*On-corhynchus mykiss*) 4-10 g	15 ºC	↑ vaccine efficacy ↑ antigen uptake by skin ↑ antigen delivery to the kidney & spleen	Nakanishi et al. (2002)
13	Live *Flavobac-terium psychrophilum*	*Flavobacterium psychrophilum*	Rainbow trout (*On-corhynchus mykiss*) 1.2-2.2 g	10-11 ºC Immersed 30 min	↑RPS	Lorenzen et al. (2010)
14	Propiscin (anaesthesia)	*Yersinia ruckeri*	Rainbow trout (*On-corhynchus mykiss*) 30-40 g	12 ± 1 ºC	↑ Immunoglobulin secreting cell (ISC) ↑ Antibody secreting cell levels (ASC) ↑ Total Ig levels ↑ Specific antibody titres ↓ Cumulative mortality rates	Siwicki et al. (2002)
15	Freund's incomplete	*Edwardsiella tarda*	Japanese flounder (*Paralichthys oliva-*	22 ºC	↑RPS ↑ Specific serum antibodies	Jiao et al. (2010)

	adjuvant, aluminum adjuvant		*ceus*) 10 g		↑ humoral immunity (aluminum) ↑ humoral & innate cellular immunity (FIA)	
16	Trypsin	*Yersinia ruckeri*	Atlantic salmon (*Salmo salar*) 2 g	15 ºC	↑ survival rates ↓ gill mucus lysozyme (6 weeks post vaccination challenge)	Costa et al. (2011)
17	*Ocimum sanctum* leaves	*Aeromonas hydrophila*	Tilapia (*Oreochromis mossambicus*) 25 g	28 ± 1.5 ºC	↑ antibody production ↑ activation of neutrophils	Logambal et al. (2000)
18	Garlic, Allium sativum	*Aeromonas hydrophila*	Rohu (*Labeo rohita*) 10 ± 2 g	28 ± 1 ºC	↑ RPS ↑ lysozyme activity ↑ Serum bactericidal activity	Sahu et al. (2007)

The incorporation of vaccines into feed to enable oral delivery to fish is desirable because it is stress free; however, there are some disadvantages. These include: short term stability of vaccines when they are mixed with feed (Komar et al., 2006), the difficulty in determining the exact dose of antigen received by each fish (Plant & LaPatra, 2011), and the destruction of the vaccine in the fish's digestive system (Polk et al., 1994). Oral immunisation has reported inconsistent results and relatively lower levels of protections than injection or immersion immunisation (Joosten et al., 1997; Newman, 1993). The first successful vaccination of trout against yersiniosis used a *Y. ruckeri* bacterin and was administered orally (Klontz, 1963). Oral booster vaccination is preferred to improve duration of protection and reduce stressing the fish. This process protects the vaccine during gastrointestinal transit until uptake by the fish hindgut (Gomez-Gil et al., 1998; Joosten et al., 1995). Other factors such as vaccine concentration, the nature of the antigen (particulate or soluble), the length of vaccination time, the size of the fish and water temperature can have great impact on antigen uptake and vaccine efficacy (Ellis, 1977; LaFrentz et al., 2014; Nakanishi & Ototake, 1997; Valdenegro-Vega et al., 2013).

Antigen uptake and duration of protection is affected by the duration of immersion and the antigen concentration (Nakanishi & Ototake, 1997). In general, shorter duration of immersion and higher dilutions tends to be less effective. For example, Tatner (1987) demonstrated that there were no significant differences in uptake of a 1/10 dilution of a vaccine at immersion times between five seconds to 10 minutes (Tatner, 1987). However, at a 1/100 dilution, there was significantly lower uptake after 100 seconds to 10 minutes of immersion compared to uptake following a two-hour immersion. Tatner and Horne (1985) reported that using very long exposures from one to six hours at very low vaccine concentrations (1:2000 and 1:5000) gave good protection.

The size of fish at vaccination influences the duration and the level of immunity. Johnson et al. (1982) reported that 1 g was the minimum size that salmonids could be effectively immunized by immersion administration using *Y. ruckeri* and *V. anguillarum* bacterins. In general, longer duration of protection occurred in larger fish, but

different responses were demonstrated in several species. In brook trout fry, the survival rate 60 days after an infectious pancreatic necrosis virus challenge was highest in two and three weeks post-hatch fry and decreased as fish increased in age or size after a single direct immersion (Bootland et al., 1990). The reason for this successful immunization of a small size fish remains unknown.

Water temperature has an effect on antigen uptake in immersion vaccination. Tatner and Horne (1983) reported that *Vibrio anguillarum* vaccine uptake decreased significantly at 5 ^{o}C compared to 18 ^{o}C in rainbow trout. Similarly, there was significant protection of vaccinated fish kept at 15 °C but no protective effect of vaccination was observed in rainbow trout reared at 5 °C and 25 °C using a bacterin of *Y. ruckeri* (Raida & Buchmann, 2008).

Vaccine efficacy is commonly evaluated using relative percent survival (RPS) which is calculated according to a formula which depends on the relationship of survival of vaccinated fish to survival of unvaccinated controls, RPS = (1 - % mortality in vaccinated/% mortality in control) × 100 (Amend, 1981). RPS of 70% - 100% are common for immersion bacterin-based yersiniosis vaccines and depend on the infection challenge dose, the size of fish, the species of fish and the duration of booster or post-vaccination (Bridle & Nowak, 2014). An alternative method of evaluating vaccine efficacy measures antibody levels in fish serum after vaccination and post infection challenge by enzyme-linked immunosorbent assay (ELISA) (Chettri et al., 2015a). Correlation between protection and antibody levels, measured by ELISA, was used in evaluating the efficacy of vaccines in Atlantic salmon and Atlantic halibut (*Hippoglossus hippoglossus* L.) (Bricknell et al., 1997; Gudmundsdóttir et al., 2003; Midtlyng, 1996). Immune gene expression was also suggested to be useful as a potential measure of vaccine success (Bridle et al., 2012). Changes in expression of 17 genes involved in an immune response, detoxification and repair was proposed as a biosignature to predict vaccine-induced protection in immersion vaccination against yersiniosis of Atlantic salmon (Bridle et al., 2012).

Yersiniosis is a common problem in salmonid hatcheries. Since yersiniosis likely affects fish from very early stages of development, it is important to vaccinate as soon as possible. However, some studies have shown that fish below 1 g had a poor or delayed response to vaccination, possibly due to undeveloped of immune systems. Long term protection was only attained when fish above 2 g were vaccinated (Amend & Johnson, 1980; Brudeseth et al., 2013).

1.3. YERSINIOSIS IN ATLANTIC SALMON

Yersinia ruckeri is a pathogen which causes significant losses in farmed salmonids (Tobback et al., 2009) and is the causative agent of both enteric red mouth disease in rainbow trout in the Northern Hemisphere (Tobback et al., 2009) and yersiniosis in Atlantic salmon (*Salmo salar*) in the Southern Hemisphere (Carson & Wilson, 2008). This pathogen is found in fish populations in Europe, North American, South Africa and Australia (Tobback et al., 2007). In Australia, there are two biotypes of *Y. ruckeri*, including serotype O1b (biotype 1) that is the predominant isolate in Atlantic salmon culture and serotype 01, non-O1b (biotype 2) which has been isolated on a few occasions and only in Tasmania (Carson & Wilson, 2009). In addition to fish, *Y. ruckeri* can be found in freshwater invertebrates (crayfish), bird faeces, humans and mammals and can survive several months in the water (Siwicki et al., 2005). *Y. ruckeri* was initially isolated in the Hagerman Valley of Idaho, USA from rainbow trout (*Oncorhynchus mykiss*) in the 1950s (Ross et al., 1966). This bacterium is a Gram-negative rod with a diameter of approximately 0.75 µm and its length varies from 1.0 to 3.0 µm (Tobback et al., 2007). It belongs to the Enterobacteriaceae family, is oxidase negative, glucose fermentative and nitrate reductive (Ross et al., 1966). *Y. ruckeri* is positive for production of β galactosidase, ornithine decarboxylase and lysine decarboxylase, whereas indole and H_2S are not induced (Tobback et al., 2007). *Y. ruckeri* colonies, are off-white, opaque, approximately 2 - 3 mm in diameter and appear after incubation for 48 h at 25 ^{o}C. The pathogen can be identified by a biochemical test (Carson & Wilson, 2009), or serological tests with ELISA, immunofluorescence antibody technique (IFAT), agglutination test (Smith et al., 1987) or PCR assay (Gibello et al., 1999).

1.4. IMMUNE RESPONSE AGAINST *YERSINIA RUCKERI*

In Atlantic salmon and rainbow trout, the innate and adaptive components of the immune response against yersiniosis have been investigated (Afonso et al., 1998; Bridle et al., 2011; Costa et al., 2011). A study on the innate immunity of Atlantic salmon affected by *Y. ruckeri*, demonstrated that lysozyme levels in gill mucus and serum bactericidal activity were increased after a *Y. ruckeri* challenge (Costa et al., 2011). Phagocytosis and killing of invading pathogens are important antibacterial defence mechanisms (Tobback et al., 2007). Phagocytes cells including neutrophils and macrophages engulf bacteria and kill them by stimulated production of reactive oxygen species (Tobback et al., 2007). Afonso et al. (1998) found that numbers of macrophage peak at 5 days and neutrophil numbers peak at 24-48 hours post injection of live or killed *Y. ruckeri* in rainbow trout. The increase in macrophages and neutrophil numbers indicated phagocytes played a role in killing the bacteria.

Host immune responses against *Y. ruckeri* have been studied at the transcription level using real-time RT-PCR to measure expression of immune-related genes (Bridle et al., 2011; Raida & Buchmann, 2007; Raida & Buchmann, 2009). Cathelicidins, a family of antimicrobial peptides, have a critical role in the innate immune defence against invasive bacterial infection (Zanetti, 2004). An in vivo challenge with *Y. ruckeri* showed the increased expression of cathelicidin 1 and 2 (asCATH1 and asCATH2) mRNA in Atlantic salmon (Bridle et al., 2011). This indicated that Atlantic salmon cathelicidins involve in an interplay between the innate and adaptive immune systems to respond more effectively to invading pathogens. In rainbow trout, an i.p. injection with *Y. ruckeri* elicited significantly increased gene expression of the pro-inflammatory cytokines IL-1b, IL-6 and TNF-a, serum amyloid protein a (SAA) and down-regulation of complement factors (C3, C5 and factor B) (Raida & Buchmann, 2009).

Immunoglobulins act as a critical part of the humoral adaptive immune response and antibody responses against *Y. ruckeri* in Atlantic salmon have been reported by various studies. IgM, the main immunoglobulin involved in systemic immune responses in

yersiniosis, has primarily been measured in serum of infected fish. Several research groups demonstrated that rainbow trout produce specific antibodies against *Y. ruckeri* following immersion vaccination (Anderson et al., 1979b; Olesen, 1991; Raida et al., 2011). In contrast, Chettri et al. (2015a) found low serum antibody levels in vaccinated groups following immersion vaccination but much higher antibody levels after challenge which indicated priming of memory cells or B-cells from the initial immersion. IgT, also called IgZ was discovered in some species after analysis of the genomes of several teleost fish species (Danilova et al., 2005; Hansen et al., 2005). While the IgM isotype has a predominant role in systemic immune responses, the IgT isotype plays a specialised role in gut mucosal responses in rainbow trout (Xu et al., 2013; Zhang et al., 2010). The gene expression of IgT in spleen has been shown to increase 10-fold after bath vaccination with *Y. ruckeri* bacterin (Raida & Buchmann, 2008). However, IgT was very weakly expressed or not regulated after intra-peritoneal vaccination with *Y. ruckeri* bacterin (Raida & Buchmann, 2007). IgT isotype has been characterised at the transcriptional level in Atlantic salmon (Tadiso et al., 2011).

1.5. YERSINIOSIS IN FARMED ATLANTIC SALMON IN TASMANIA

Atlantic salmon farming reached Australia in the mid 1980s (Darby, 2003). Australian salmonids production increased by 222% in value ($343 million) and by 179% in production volume (27,553 tonnes) from 2002-03 to 2012-13, and Tasmanian accounted for much of the strong growth (Stephan & Hobsbawn, 2014). In Tasmania, salmonids are the largest aquaculture species group, accounting for 90% of the volume and 94% of the value of Tasmania's aquaculture production in 2012-13 (Stephan & Hobsbawn, 2014).

Yersiniosis, or enteric redmouth disease (ERM), was first described in rainbow trout in 1955 (Rucker, 1966). Yersiniosis is now widespread in farmed populations of a variety of salmonid and other species (e.g. eel, goldfish, perch, channel catfish, sole, sturgeon and turbot) in the USA, Europe and Australia (Carson & Wilson, 2009). Yersiniosis can affect all sizes of fish, but tends to occur as a less severe but more chronic form in larger

fish (Furones et al., 1993). When fish are exposed to *Y. ruckeri*, first mortality occurs approximately 4-8 days later, and can increase up to 50-70% during a typical 30-60 days course of clinical infection (Busch, 1978). In most countries, yersiniosis is mostly a disease of rainbow trout, but in Australia it occurs predominantly in farmed Atlantic salmon (Bridle & Nowak, 2014; Carson & Wilson, 2009). The Hagerman strain, which causes ERM in rainbow trout, is exotic to Australia. (Bridle & Nowak, 2014).

The first commercial yersiniosis vaccine was produced and licensed in 1976 as formalin-killed whole cells of *Y. ruckeri* (see Bridle et al., 2012). In Australia, Yersinivac-B was developed by Department of Primary Industry Water and Environment Tasmania. Currently, Yersinivac-B is a commercial bacterin-based vaccine manufactured by MSD Animal Health and prepared from formalin-killed whole cells of Australian serotype O1b. It is used for immersion vaccination for most Tasmanian Atlantic salmon which until recently, were vaccinated once by bath immersion at 5 g. However, significant mortality due to yersiniosis still occurred and the greatest fish loss in a single Tasmanian hatchery occurred in 2007 when half a million vaccinated juvenile Atlantic salmon died (Costa et al., 2011). Currently, Tasmanian Atlantic salmon are vaccinated when they weigh 1-3 g and then again at 5 g, as a booster. However, mortalities due to *Y. ruckeri* still occur in farmed Atlantic salmon, particularly amongst small fish in hatcheries. There is a need to develop better vaccination strategies to prevent mortality and morbidity in Atlantic salmon due to yersiniosis.

1.6. AIM AND THESIS STRUCTURE

This thesis focused on the improvement of yersiniosis vaccine efficacy for Atlantic salmon by using different administration methods of immersion immunisation. To address this objective, this thesis has the following aims:

- Evaluate the effects of different inactivation methods of *Y. ruckeri* on the efficacy of single dip vaccines including formalin inactivation, ammonium sulphate inactivation, and pH-lysed then formalin treated inactivation.

- Compare hyperosmotic pretreatment with direct immersion in Atlantic salmon (*Salmo salar*) by using an ammonium sulphate inactivated *Y. ruckeri* whole-cell vaccine.

- Investigate the vaccine performance of immersion vaccination with single dip and double dip vaccination of small fish at hatchery stage.

This thesis has five chapters in total and is structured as follows:

Chapter 1 (this chapter) contains a general introduction to the immune response of teleosts, general knowledge about fish vaccination, the pathogen studied, the immune response of fish against *Yersinia ruckeri*, the effects of yersiniosis with farmed Atlantic salmon in Tasmania and the thesis aims.

Chapter 2 describes the results obtained from an experiment with different inactivation methods of *Y. ruckeri* where the vaccine efficacy of Atlantic salmon were determined at the relative percent survival (RPS), antibody production levels, carrier status of *Y. ruckeri* and gene expression.

Chapter 3 describes the results of an experiment where the vaccine efficacy of ammonium sulphate inactivated bacterin combined with hyperosmotic infiltration in vaccination was assessed.

Chapter 4 describes the results of an experiment where the vaccine efficacy was investigated with single dip and double dip immersion vaccination at first feeding.

Chapter 5 is general discussion that assembles the most significant findings from the research chapters and connects them with current knowledge of the vaccine prevent yersiniosis.

CHAPTER 2.

THE EFFECTS OF INACTIVATION METHODS OF *YERSINIA RUCKERI* ON THE EFFICACY OF SINGLE DIP VACCINATION

2.1 INTRODUCTION

The method of inactivation of the pathogen can have a significant effect on vaccine efficacy. Commonly used methods for inactivating bacterial cells include treatment with formalin, chloroform, lysis at pH 9.5, disruption with sodium dodecyl sulphate (SDS), heat and sonication (Austin, 1984). Formalin inactivated bacterial pathogens are most commonly used in vaccine preparations (Sommerset et al., 2005). Inactivation of bacteria by formalin influences the physico-chemical characteristics of surface antigens and may reduce protective efficacy against pathogenic bacteria (Tu et al., 2010). Inactivating *Y. ruckeri* by chloroform instead of formalin did not improve performance of a vaccine for rainbow trout (*Oncorhynchus mykiss*) (Amend et al., 1983). However, *Y. ruckeri* bacterin prepared using pH lysed cells at pH 9.8 for 60 to 120 min had significantly improved efficacy (Amend et al., 1983). Ammonium sulphate, due to its kosmotropic properties, is commonly used to precipitate proteins (salting out) (Lovrien & Matulis, 2001). Ammonium sulphate has been used successfully to purify immunogenic proteins (Dzata et al., 1991), leukotoxin (Confer et al., 2006) and egg yolk immunoglobulin (Zhen et al., 2008). RNAlater used to preserve DNA, RNA and protein indefinitely when stored at -20 ^{o}C (Ambion, Life Technologies) is predominantly saturated ammonium sulphate (70%). Due to the ability to precipitate and preserve antigens, ammonium sulphate has the potential as an inactivating agent for vaccine production.

Vaccine efficacy is normally evaluated using the relative percent survival (RPS), cumulative percent mortality, antibody levels in fish serum after vaccination or expression of immune-relevant genes. The differential expression of specific genes linked to immune response, detoxification and repair were proposed as a biosignature for prediction of induced protection in Atlantic salmon immersion-vaccinated against yersiniosis (Bridle et al., 2012). This proposed biosignature involved upregulation of 14 genes and downregulation of three others. According to the selection criteria proposed by Bridle et al. (2012), nine genes were suitable for evaluation and were selected for analysis (genes chosen described section 2.2.7). The nine genes were representative of highly upregu-

lated genes, medium upregulated genes and downregulated genes. The hepcidin gene was selected as this peptide has potential activity against bacteria (Hu, 2008) and plays an important role in inflammatory response (Bao et al., 2005). It is also known to reduce splenomegaly and regulate iron homeostasis in mice in relation to immune functioning (Gardenghi et al., 2010). LIM and actin-binding protein are related to immunity and known to enhance bacterial internalisation in mammals (Pizarro-Cerdá & Cossart, 2006). The fish virus induced TRIM protein is also related to diverse receptors of the host immune system and immune recognition of pathogens (Ellis, 1989). The expression of myelin and lymphocyte protein has been implicated and was also regulated in mammalian T-cell development (Frank, 2000). The bifunctional 3-phosphoadenosine 5-phosphosulfate synthetase 2 gene was related to antioxidation activity and xenobiotic metabolism (Sahlmann et al., 2013).

Even after vaccination, there is a possibility that some fish may harbor *Y. ruckeri* without showing clinical signs (i.e. asymptomatic carriers) (Bridle & Nowak, 2014; Fadaeifard et al., 2014). Rodgers (1992) found *Y. ruckeri* in the faeces of carrier fish two months after the disease outbreak. *Y. ruckeri* can remain viable in the kidney and the intestinal tract of the carriers and cyclical shedding of *Y. ruckeri* can occur. This can be exacerbated when fish are under stressful conditions, resulting in reinfection and spreading of yersiniosis (Busch & Lingg, 1975; Carson & Wilson, 2009). Further investigation of the presence of *Y. ruckeri* in vaccinated fish is needed to understand the reservoirs of the pathogen and risk factors for infection post-vaccination.

This study was designed to determine the effects of different inactivation methods for *Y. ruckeri* on the efficacy after single dip bacterin administration. These methods included formalin inactivation, ammonium sulphate inactivation, and pH-lysed then formalin treated inactivation. This study aimed to investigate whether ammonium sulphate can be used for *Y. ruckeri* inactivation and can enhance protection compared to conventional formalin-killed and pH-lysed bacteria. Specific antibody levels were investigated as a measure of adaptive immune response in Atlantic salmon serum after administration of each bacterin preparation. The use of gene expression as a biosigna-

ture of vaccine performance was evaluated. Spleen samples were analysed for presence of *Y. ruckeri* to determine carrier status.

2.2 MATERIALS AND METHODS

2.2.1 Fish

Atlantic salmon (*Salmo salar*) with an average body weight of 5 g were provided by Salmon Enterprises of Tasmania (SALTAS) and held at the Aquaculture Centre, University of Tasmania. The fish were acclimated to laboratory conditions for five weeks prior to the experiment. Fish were held in flow through fresh water holding tanks of 4000 L at 15 ^{o}C and then moved to five 1500 L tanks after vaccination. Salmon were fed daily to satiation with a commercial diet (Skretting). Before vaccination and challenge, two to three fish from each treatment were lethally anaesthetised with clove oil (1 mL in 1 L of water) and checked for the presence of *Y. ruckeri* by culturing kidney samples on blood agar plates. All samples were negative of *Y. ruckeri* before vaccination and challenge. Water quality, including temperature, pH, ammonia, and chlorine, were assessed daily. This experiment was approved by the University of Tasmania Animal Ethics Committee (Approval Number A0012285).

2.2.2 Preparation of bacterins

A strain of *Y. ruckeri*, UTAS001, was obtained from the Institute for Marine and Antarctic Studies (IMAS), UTAS culture collection, and used to prepare the bacterin and challenge inoculation. A 300 mL brain heart infusion (Oxoid, England) starter broth was inoculated and shaken for 24 h, then 100 mL of this starter was added to a 10 L Nalgene container and cultured for 46 h at room temperature. The number of bacterial cells was counted using a Neubauer hemocytometer after inactivating the cells with 10% formalin.

Three different inactivation methods were used: formalin inactivation, ammonium sulphate inactivation then heat, and pH-lysed then formalin treated inactivation. Formalin was added to 2×10^9 cells/mL to achieve a 0.3% final concentration for inactivation.

Ammonium sulphate inactivation was achieved by adding ammonium sulphate to the bacterin stock to achieve a 50% solution (1 kg ammonium sulphate into 2 L bacteria) and then adjusting the pH from approximately 7.2 to 5.2 with concentrated hydrochloric acid (HCl), as measured by a digital pH meter. This vaccine was then incubated at 60 ^{o}C for 2 h. The pH-lysed experimental vaccine was prepared by adjusting the pH to 10 and leaving the bacterial culture for 3 h before adjusting the pH from approximately 10 to 7.4, and inactivating with 0.3% formalin. After the inactivation, a small sample of each bacterin was inoculated on both blood agar plates and a LB broth base (Sigma, USA) to confirm inactivity. The absence of any bacterial growth after 24 h indicated the sterility. The bacterins were stored at 4 ^{o}C until required.

2.2.3 Vaccination

One hundred and fifty fish (8 to 10 g) were used for each treatment. During vaccination, the fish were netted and dipped into a 20 L bucket containing 2 L of a 1:10 dilution of one of the three vaccines for a period of 60 s (dipping 50 fish/time). After vaccination, the fish were dipped in water to remove excess vaccine. The control fish were dipped in water for 60 s with no added bacterin (150 fish). The positive control fish (125 fish) were vaccinated by intraperitoneal injection (100 µL/fish) with the formalin inactivated bacterin. All vaccine preparations started with the same concentration of bacteria.

2.2.4 Challenge

At 12 weeks post vaccination, fish from each treatment (n=75) were randomly selected and transferred to a challenge room and challenged with *Y. ruckeri* (9×10^{5} colony forming units (CFU)/mL for 1 h) in air saturated 20 L buckets with fresh water at 15 ^{o}C. The fish were then rinsed in fresh water and transferred into 200 L recirculating tanks (3 tanks per each of the five treatments). Moribund fish were removed and recorded daily until the end of the experiment. Relative percentage survival (RPS) was calculated, RPS = (1 – (% mortality/% control mortality)) x 100 at the end of the experiment (Amend, 1981). For moribund fish, bacteria were re-isolated from two to

three fish per treatment and identified for confirmation of cause of morbidity and disease.

2.2.5 Sampling

From each treatment, 10 fish were sampled before vaccination (time 0), at week 6 and at week 12 post-vaccination. Sampled fish were anaesthetised with clove oil (1 mL in 15 L of water) and blood plasma was collected from the caudal vein using EDTA (pH 8.0) as an anticoagulant. Due to undetectable antibody results of ELISA assay with blood plasma at week 6, blood serum was collected from whole blood without EDTA at week 12 post-vaccination after clot formation overnight at 4 ^{o}C. Plasma or serum was recovered by centrifuging at 5000 x *g* for 10 min at 4 ^{o}C and stored at -20 ^{o}C. Gills were collected and stored in an RNA preservation solution (25 mM sodium citrate, 10 mM EDTA, 70 g ammonium sulphate/100 mL solution, pH 5.2) at 4 ^{o}C overnight before transfer to -20 ^{o}C. At the end of the experiment, 20 survivors were sampled from each treatment to determine carrier status using Quantitative real-time RT-PCR (qPCR).

2.2.6 Blood analysis

2.2.6.1 Indirect Enzyme-linked immunosorbent assay (ELISA)

ELISA was used to detect the presence of specific antibodies in the blood serum using lipopolysaccharide (LPS) extracted from *Y. ruckeri* as a coating antigen. For LPS extraction, 350 mg (wet weight) formalin-inactivated cells of *Yersinia ruckeri* were placed into a 50 mL tube and then centrifuged at 3,000 x *g*, 4 ^{o}C for 30 min. The supernatant was discarded and then 2.2 mL of distilled water was added to the pellet. The pellet was then transferred to a 15 mL tube and 0.4 mL of 100 mM Tris-HCl (pH 8.0), 0.4 mL of 0.5 M $MgCl_2$ and 1.0 mL of 8% Triton X-100 were added, and the tube then heated in boiling water for 10 min. After cooling, the resulting mixture was centrifuged at 15,000 x *g* (max, fixed angle) for 15 min and then the precipitate (or pellet) was washed once with 4 mL of 10 mM Tris-HCl (pH 8.0)-10 mM $MgCl_2$. The tube was then centrifuged at 15,000 x *g* (max, fixed angle) for 15 min and the superna-

tant discarded. Then 1mL each of distilled water, 0.2 M EDTA (pH 8.0), 2 M NaCl and 8% Triton X-100 was added to the precipitate and mixed well. This suspension was incubated at 37°C for 60 min with gentle agitation, and then centrifuged at a fixed angle at max (15,000 x *g*) for 15 min. The supernatant was removed and put in a fresh 15 mL tube. To this tube, 0.6 mL of 1 M $MgCl_2$ was added and 1 mL of 100% ethanol was then followed by the drop-wise addition. This tube was then incubated at 37°C for 60 min. After incubation, this sample was immediately centrifuged at 20 ^oC maximum at a fixed angle (18,514 x *g*) for 60 min and the supernatant discarded. The transparent precipitate was washed once with 4 mL of 10 mM Tris-HCl (pH 8.0)-10 mM $MgCl_2$, and centrifuged again at 20 ^oC maximum at a fixed angle (18,514 x *g*) for 5 min. Finally, the LPS pellet was weighed, resuspended in distilled water and then used for ELISA.

ELISA was used to detect the presence of specific anti-*Yersinia* LPS antibodies in the blood serum. The monoclonal anti-salmonid Ig (H chain) antibody was supplied by Cedarlane Laboratories Ltd, product number CLF004HP, and all wash buffers were prepared according to their protocols. 96-well flat bottom ELISA plates (Iwaki, Science Product Dept., Asahi Glass Co., Ltd.) were coated by 100 µL dilution of 10 µg/mL LPS antigens, *Y. ruckeri,* in a coating buffer and left overnight at 4 ^oC. Three washes with a low salt wash buffer removed the coating solution. Free binding sites were blocked by 2 h incubation with 3% skim milk at 18 ^oC and then washed three times with a low salt wash buffer. After washing, 100 fold dilutions of serum in PBS were added in duplicates at 100 µL/well. A positive standard sample which consisted of a pool of sera from fish immunised with Yersinivac-B by i.p. from a previous experiment, aliquoted and maintained at -20 °C. This standard with the dilutions of 1:100, 1:200, 1:400, 1:800, 1:1600 and 1:3200 were added to "standard wells" in triplicate at 100 µL/well. Two wells of every plate were blanks. Plates were then sealed and incubated in a plate shaker for 2 h at 18 ^oC. A high salt wash buffer was used to wash the plates three times with 5 min incubation at room temperature for the last wash. 100 µL of reconstituted monoclonal anti-salmon Ig – HRPO (Horseradish Peroxidase) conjugated (Cedarlane Laboratories Ltd, product number CLF004HP) with 500 fold dilutions was added to

each well and incubated at 18 ºC for 1 h. After incubation, the plates were washed five times with a high salt wash buffer, with 5 min incubation at room temperature for the last wash. Chromogen was then added (100 µL/well of TMB One Solution, Promega G7431) to each well and after 10 min incubation at room temperature, a stop solution (100 µL/well of 1 M sulphuric acid) was added. The plates were read at 450 nm (Spectra Rainbow Thermo, Xread Plus V4.04, Serial 9340045) after being shaken for 10 seconds. Absorbance values were referred to an arbitrary unit (AU) of 100 AU for 100 fold dilutions, 50 AU for 200 fold dilutions, 25 AU for 400 fold dilutions, 12.5 AU for 800 fold dilutions, 6.25 AU for 1600 fold dilutions and 3.125 AU for 3200 fold dilutions. A standard curve was generated by using the four parameter logistic curve (SigmaPlot 11.0) and plotting the AU of the hyper-immune serum against absorbance and converted absorbance sample values to AU.

2.2.6.2 Bacterial agglutination

Agglutination activity against *Y. ruckeri* in fish serum samples was determined using a modified version of a method described by Roberson (1990). Firstly, 25 µL of sterile PBS was added to all wells of a 96 well U-bottomed microtiter plate. Then 25 µL of serum were added to the first column of this plate with duplicate rows. A twofold dilution series was then made across each row (from 1:2 dilution to 1:4096 dilution). A 25 µL aliquot of the bacterial suspension (1×10^9 cells/mL) that was fixed in 0.5% formalin, centrifuged at 2000 x *g* at 4 ºC for 10 min, and washed three times with sterilised phosphate buffered saline (PBS) was then added to all wells. The plate was shaken for 30 seconds and incubated overnight at 18 ºC. A circular mat above settled bacteria represented a positive agglutination, while a non-turbid solution with a central settle pellet indicated a negative agglutination. The agglutination titre was determined as a reciprocal of the highest serum dilution showing agglutination.

2.2.7 Quantitative Real-time PCR Analysis

A total of 20 fish including 10 fish of naive unvaccinated (week 0) and 10 fish of ammonium sulphate inactivation vaccine (week 6), were used to analyse the expression of nine genes of interest (Table 2.1).

Table 2.1 List of nine genes chosen for gene expression analysis in the gills of Atlantic salmon before and after ammonium sulphate inactivated vaccine

Accession number	Gene
EG929305	Uncharacterized protein KIAA1033
DY699380	LIM and actin-binding protein 1 [*Salmo salar*]
DY729690	Hepcidin
EG859007	E3 ubiquitin-protein ligase Itchy
CA054083	Immunoglobulin mu heavy chain [*O. mykiss*]
DW565729	Fish virus induced TRIM protein [*O. mykiss*]
CB496376	Myelin and lymphocyte protein
EG912256	Bifunctional 3-phosphoadenosine 5-phosphosulfate synthetase 2
EG779342	Thioredoxin interacting protein [*Salmo salar*]

2.2.7.1 RNA extraction, DNA decontamination and reverse transcription

Gill samples (5-10 mg, approximately 0.5 cm x 0.5 cm) were removed from RNA preservation reagents and cells were lysed using 400 µL of extraction buffer (4 M urea, 1% SDS and 2 X PBS) supplemented with 5 µL (5 units) of proteinase K. The resulting suspension was cooled on ice for 5 min and incubated at room temperature (RT) for 60 min with occasional mixing by vortex. Protein was removed by precipitation with the addition of 1mL of TRI-Reagent (Sigma-Aldrich) followed by 150 µL of BCP (bromochloropropane) and centrifuged at 12,000 x *g* for 15 min at 4 ^{o}C. RNA was then precipitated from the supernatant with the addition of one volume of isopropanol with pink-pellet paint co-precipitate (1:500 Pink: Iso) and centrifugation at 16,000 x *g* for 10

min at RT. The nucleic acid pellet was then washed twice with 75% ethanol and eluted in 40 µL of water with 20 mM DTT at 55 ^{o}C for 5 min. Removal of residual genomic DNA was achieved by treatment of the purified RNA with 2 units of Baseline-ZERO™ DNase (Epicentre) for 30 min at 37 ^{o}C. Total RNA concentration was measured by using a Qubit® 2.0 Fluorometer (Life Technologies) and Quant-iT RNA assay kit (Invitrogen, VIC, Australia). The integrity and purify of RNA was examined by running in 1% agarose gel using RNA EZvision dye (Amresco, Sydney, Australia) as per manufacturer's instructions. RNA was reversely transcribes using a 50 µM of Oligo dT18 primer (0.8 µL per sample typical stock, 1 µL of 10 mM dNTP, 2 µL of 5 X RT buffer, 0.25 µL of RNAse inhibitor, 6.25 µL of molecular grade water and 0.5 µL of reverse transcriptase) and random Hexomer 50 ng/µL (0.2 µL per sample typical stock). Reactions were performed in a C1000 thermo cycler (Bio-Rad, Australia) with 20 µL reaction volumes containing 2 µg of total RNA extraction (9 µL samples). The reactions were run under the following conditions: 65 ^{o}C for 10 min, 42 ^{o}C for 50 min, 70 ^{o}C for 15 min and 12 ^{o}C indefinitely.

2.2.7.2 cDNA Template

Following cDNA generation, five standards were created, 3 µL of each sample was pooled into a single eppendorf tube and diluted with 2 volumes of molecular grade water to create standard 1. The other four standards were made by serially diluting in 4-fold dilution with water (i.e. 30 µL into 90 µL of water, the highest to the lowest). The remaining samples were diluted with water in 4-5 folds (i.e. 73 µL of H_20 added to the 17 µL of the remaining samples after the standard pooling, making the total volume of 90 µL). All samples and standards were then transferred to the cDNA plate and held at 4 ^{o}C for later use.

2.2.7.3 Real-Time qPCR for Gene Expression Analysis

A single master mix was prepared for all samples and standards by mixing 3.96 µL of water, 0.02 µL of 100 mM forward primer, 0.02 µL of 100 mM reverse primer and 5 µL of 2X polymerase/syber mix (i.e. SensiFast) per reaction. This master mix was then transferred to a qPCR plate (9 µL/well) in a Bio-Rad iQ5 Real-Time machine (Bio-

Rad) by using a multichannel pipette. Next, 1 µL of cDNA template from a stock plate was transferred into this qPCR plate and mixed by using a pipette. A film seal was applied to the plate and the plate was then short spun to 500 g at 4 ^{o}C. A run protocol and the plate setup were selected using real-time software. A generic protocol for SensiFast was: 94 ^{o}C for 2 min, then 95 ^{o}C for 5 sec, 55 ^{o}C for 20 sec and 72 ^{o}C for 10 sec for 40 times and then for melting curve 95 ^{o}C for 1 min, 55 ^{o}C for 1 min and 55 ^{o}C + 1 ^{o}C/10 sec for 36 times, until 90 ^{o}C. An amplification efficiency of the standards should generate between 85-105% with an R^2 higher than 0.98. This real-time qPCR was used to measure expression of the nine different genes of uncharacterized protein KIAA1033, LIM and actin-binding protein, hepcidin, E3 ubiquitin-protein ligase Itchy, immunoglobulin mu heavy chain, fish virus induced TRIM protein, myelin and lymphocyte protein, bifunctional 3-phosphoadenosine 5-phosphosulfate synthetase 2 and thioredoxin interacting protein. When the amplification efficiency of the standards of the genes generated between 85-105%, mRNA expression levels were determined by qBase Plus software (Biogazelle, Belgium) using the mean expressions of two reference genes – elongation factor 1a (EF1a) and b-actin. qBase Plus software was also used for analyse the qPCR data including statistical analysis (ANOVA) and fold change of difference genes.

2.2.8 Carrier status analysis

2.2.8.1 Total nucleic acid (TNA) extraction from spleens

A 10-20 mg sample of spleen was removed from RNA preservation reagents and TNA extracted by addition of 600 µL of extraction buffer (4 M Urea, 1% SDS, 0.2M sodium chloride, 1 mM sodium citrate, pH 8.2) supplemented with 5 µL of proteinase K. The resulting suspension was incubated at 37 ^{o}C overnight with occasional mixing by vortex. Protein, cellular debris, and detergent were then removed by centrifugation in 300 µL of 7.5 M ammonium acetate at 16,000 x *g* for 5 min at 18 ^{o}C. Nucleic acids were then precipitated from the supernatant by addition of an equal volume of isopropanol with pink-pellet paint co-precipitate (1:500 Pink:Iso) and centrifugation at 16,000 x *g* for 10 min at RT. The nucleic acid pellet was then washed twice with 70% ethanol

and resuspended in 200 µL elution buffer [molecular-grade water containing 10 µm TRIS-HCL and 0.05% Triton X100 (Sigma- Aldrich)] by incubation at 55 ^{o}C for 10 min.

2.2.8.2 PCR for carrier status

The PCR master mix consisted of 5 µL of 2 X My Taq^{TM} HS DNA Polymerase mix (Bioline), 0.4 µL each of forward primer of *Y. ruckeri* (YrF) and reverse primer of *Y. ruckeri* (YrR) (400 nM of each), 0.1 µL of *Y. ruckeri* (Yr) probe (100 nM), 2.1 µL of PCR water (Bioline BIO-37080) and 2 µL of TNA sample. The PCR amplification program was as follows: 95 ^{o}C for 3 min, then 95 ^{o}C for 10 sec and 60 ^{o}C for 30 sec for 44 cycles. 2 µL of PCR water was used as no template control. Primers and probe used for PCR were YrF (5'-AAC CCA GAT GGG ATT AGC TAG TAA-3'), YrR (5'- GTT CAG TGC TAT TAA CAC TTA ACC C -3') and Yr probe (Taqman) (5'-AGCCACACTGGAACTGAGACACGGTCC-3').

2.2.9 Statistical analysis

Survival was analysed using SigmaPlot 11.0, Kaplan-Meier Survival Analysis. Multiple comparisons were performed using the Holm-Sidak method including adjustment of the P value to avoid type I errors. A one-way ANOVA (Analysis of Variance) was used for the analysis of results from quantification of *Y. ruckeri* cells mg^{-1} of spleen between the control fish and three other vaccinated groups, and if significant followed by a Turkey's post hoc test to identify where significant differences occurred among treatment means. Difference in percentage of *Y. ruckeri* carriers was tested for significance using Chi-square analysis. The results were considered significant when $P < 0.05$. The Microsoft Excel 2010 Data Analysis Package was used to analyse correlation coefficients between antibody level and agglutination activity of serum of each vaccinated groups according to Pearson's method. Data input and statistical analysis were achieved using SPSS 22.0 software (SPSS Inc., USA).

2.3 RESULTS

Fish mortalities were observed from day 3 to day 14 post challenge. The negative control fish had a lower survival rate (50%) than the fish vaccinated with ammonium sulphate inactivated bacterin (96.7%), pH-lysed inactivated bacterin (90.62%), and formalin inactivated bacterin (92.71%) and the positive control (100%) (Figure 2.1). Survival of fish vaccinated with ammonium sulphate inactivated bacterin was not significantly different from either pH-lysed inactivated bacterin (P =0.0968) or formalin inactivated bacterin (P = 0.233).

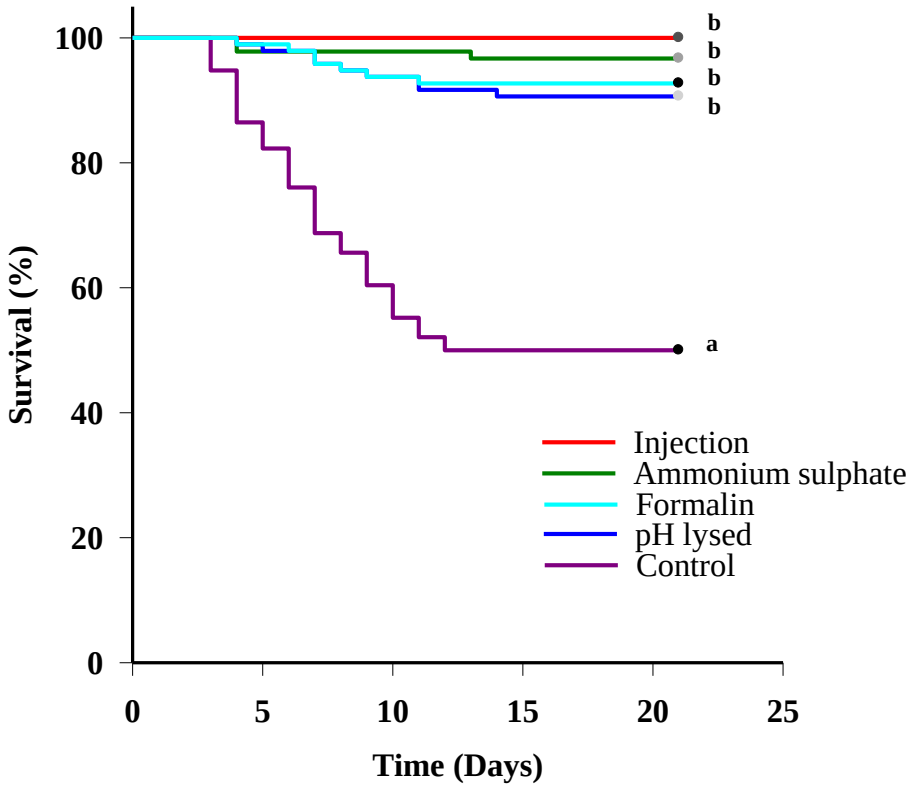

Figure 2.1 Effects of three different inactivation methods for *Y. ruckeri* bacterin preparations administered by immersion (ammonium sulphate inactivation, formalin inactivation and pH-lysed then formalin inactivation) compared to intraperitoneal injection with bacterin and unvaccinated negative control group on survival of Atlantic salmon after disease challenge. Twelve weeks post-vaccination, 63 Atlantic salmon from the injection group and 91 to 96 fish for each of the four other groups of were

challenged by immersion with 9×10^5 CFU/mL of *Y. ruckeri*. Different letters indicate significant differences (Survival analysis, SigmaPlot 11.0, $P < 0.05$)

The unvaccinated fish showed a significantly lower survival rate than the three vaccinated groups of fish and the positive control fish. The mortality in vaccinated fish varied from 3.30% to 9.38%, giving the RPS of the ammonium sulphate vaccinated fish, formalin vaccinated fish and pH-lysed then formalin treated vaccinated fish at 93.4%, 85.4% and 81.3% respectively (Table 2.2).

Table 2.2 Protection of Atlantic salmon following vaccination using bacterin produced by different inactivation methods

Treatments	**Number of fish**	**Specific mortality**	**Survival** (%)	**Mortality** (%)	**RPS** (%)
Control	96	48	50.00	50.00	0
Injection	63	0	100	0	100.0
Ammonium sulphate + heat	91	3	96.7	3.30	93.4
pH-lysed + formalin	96	9	90.62	9.38	81.3
Formalin	96	7	92.71	7.29	85.4

Only the injection vaccinated fish showed measureable serum antibody levels (week 12). Titre ranged from 2.2 to 89.1 AU, with the mean value of 13.69 ± 27.02 AU. No antibody titre was seen in the unvaccinated fish (control) or immersion vaccinated fish regardless of the vaccine used.

Bacterial agglutination was evident at a titre ranging from 4 to 128 only in the positive control fish (injection vaccination). No agglutination was seen in the unvaccinated fish serum (control), the ammonium sulphate vaccinated fish serum, the formalin vaccinated fish serum or the pH-lysed then formalin treated vaccinated fish serum.

There was no correlation between antibody level and agglutination activity of serum in Atlantic salmon against *Y. ruckeri* in the injection inactivation group with R^2 of 0.0014

(Figure 2.2). The lack of relationship ($P > 0.05$) between average antibody titre and agglutination in the injection inactivation group was confirmed by using the non-parametric Spearman r test, with a Spearman r of 0.298.

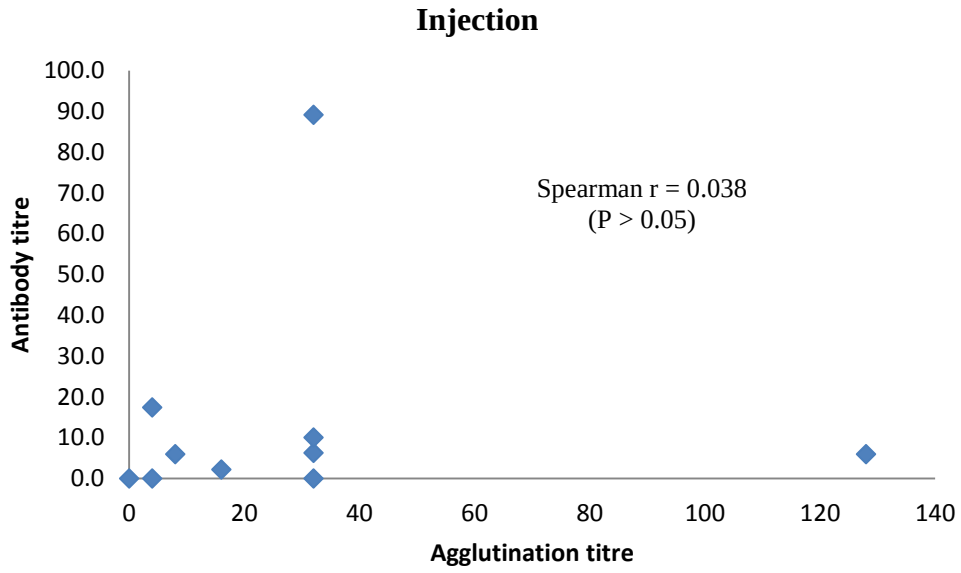

Figure 2.2 The correlation between antibody level and agglutination activity of serum in Atlantic salmon against *Y. ruckeri* in injection inactivation group

Gene expression results showed that four genes were excluded from further analyse due to their amplification efficiency of the standards outside 85-105%. These four genes were uncharacterised protein KIAA1033, E3 ubiquitin-protein ligase Itchy, immuno-globulin mu heavy chain, and thioredoxin interacting protein. The results of five of differentially expressed genes in the gills when the unvaccinated fish (week 0) and the vaccinated fish (week 6) of the ammonium sulphate inactivated vaccine were compared are shown in Table 2.3. All five genes were up-regulated in the fish vaccinated with the ammonium sulphate inactivated vaccine six weeks post-vaccination.

Table 2.3 List of differentially expressed genes in the gills of Atlantic salmon showing significant upregulation (ANOVA P < 0.05) before and after vaccination (week 0 and week 6) of the ammonium sulphate group before *Y. ruckeri* challenge. Arrows indicate the direction of the fold change

Genes	Differential regulation Week 0 vs Week 6	P value
LIM and actin-binding protein 1	3.2 ↑	0.0001
Hepcidin	2.5 ↑	0.0060
Fish virus induced TRIM protein	4.1 ↑	0.0072
Myelin and lymphocyte protein	2.5 ↑	0.0003
Bifunctional 3-phosphoadenosine 5-phosphosulfate synthetase 2	3.4 ↑	0.0001

The presence of *Y. ruckeri* in the spleen of surviving fish of five treatments at week 15 is shown in Table 2.4. The unvaccinated control fish had the highest number of fish that were carriers of *Y. ruckeri* (7/20) and the lowest number was in the groups vaccinated with the ammonium sulphate inactivated bacterin (1/20) and the formalin inactivated bacterin (1/20). The number of bacteria ranged from 0.01×10^2 to 0.83×10^2 *Y. ruckeri* cells mg^{-1} of spleen and there was no significant difference in numbers between the control fish and the four other groups ($P > 0.05$).

Table 2.4 Presence of *Y. ruckeri* cells in the spleen by qPCR at 15 week post vaccination (surviving fish). No significant difference was observed between the control fish and the four other groups (P > 0.05)

Treatment	Organ	Number of samples positive for *Y. ruckeri*	*Y. ruckeri* number mg^{-1} (mean ± SD)
Control	Spleen	7/20	83 ± 189
Injection	Spleen	4/20	11 ± 31
Ammonium sulphate	Spleen	1/20	1 ± 6
pH-lysed	Spleen	2/20	36 ± 112
Formalin	Spleen	1/20	12 ± 54

2.4 DISCUSSION

The present work showed high protection by three kinds of inactivated vaccines for Atlantic salmon against yersiniosis. There was a significant difference between unvaccinated fish (control) and the other three vaccinated groups including ammonium sulphate inactivated bacterin, pH-lysed inactivated bacterin, and formalin inactivated bacterin. The ammonium sulphate inactivated group had 8-12% higher RPS than either the pH-lysed inactivated group or the formalin inactivated group. However, no significant difference was found in these groups. Formalin is currently the most commonly used inactivation method in vaccine preparations. The cross-linking of proteins caused by formalin treatment may have a negative effect on the immunogenicity of some protein antigens or obscure epitopes (Kaminski et al., 2014; Klockenbusch & Kast, 2010; Sompuram et al., 2004). Ammonium sulphate is the most commonly used salt for salting out proteins or protein purification and can preserve potential antigens by precipitating potentially damaging proteases and nucleases as used in RNAlater, a sample preservation reagent that is 70% ammonium sulphate. Using ammonium sulphate to inactivate *Y. ruckeri* to produce vaccines may be more efficient compared to formalin inactivated bacterin due to the lack of cross-linking of proteins and may increase the shelf-life of the bacterin because of its precipitation of potentially damag-

ing proteases. To the best of my knowledge, ammonium sulphate has not been used previously for inactivation of bacteria for fish. Therefore, potential impacts or concerns over fish welfare by using this vs other methods need to be further investigated. The value of ammonium sulphate for fish vaccines in yersiniosis should be further investigated.

The vaccine that was inactivated by pH-lysis then formalin treated gave good protection (RPS = 81.3%) in this experiment. It has been shown that bacterins prepared from pH-lysed cells help to release O-antigen which significantly increases the potency of *Y. ruckeri* bacterins (Amend et al., 1983). Fish vaccine efficacy can be enhanced through the use of specific O-antigen extractions (Anderson et al., 1979a) as they improve humoral and cellular defence mechanisms in fish (Anderson & Jeney, 1991). However, the present study did not show a significant difference in the protection between pH-lysed inactivated group and the common formalin inactivated whole-cell vaccine.

Blood plasma at six week post vaccination was collected and antibody levels against *Y. ruckeri* were measured but there was no detectable antibody level in any of the groups. Therefore, at 12 week post vaccination serum was sampled following some studies on antibody response in *Y. ruckeri* vaccine (Chettri et al., 2015a; Jaafar et al., 2015; Soltani et al., 2014). This change in method for blood collection would not change the detection sensitivity because when I used blood plasma, there was no antibody level in all groups (data not show). Fish serum antibodies represent a specific humoral defence by neutralising bacterial toxins and prohibiting bacterial adherence or incursion into non-phagocytic host cells (Ellis, 1999). Antibodies against *Y. ruckeri* were found only in the injection vaccinated fish that had the highest protection against *Y. ruckeri*. The present results were consistent with the results of Chettri et al. (2015a) that found serum antibody levels of vaccinated and unvaccinated groups before challenge were low and not significantly difference after immersion vaccination with a *Y. ruckeri* vaccine. Similarly, although Kumari et al. (2013) found that antibody levels against *Aeromonas salmonicida* in turbot (*Scophthalmus maximus*) were high in injection vaccinated fish, there were virtually no antibody in immersion vaccinated fish at 28 d, 60 d, 120 d and 180 d post treatment. Previous studies have shown that trout produce

specific antibodies against *Y. ruckeri* following immersion vaccination (Anderson et al., 1979b; Olesen, 1991; Raida et al., 2011). However, detectable antibody titre was absent in unvaccinated fish (control) and all immersion vaccinated fish in the present study. The immunological mechanism behind the protective effect of the *Y. ruckeri* immersion vaccination remains unknown. Chettri et al. (2015a) reported that antibody levels were not detected after immersion vaccination with a *Y. ruckeri* vaccine but the antibody reactions were significantly elevated after challenge in these groups. One of the possible explanations could be the immersion vaccination induced priming of memory cells or B-cells that are involved in the protection after exposure to live bacteria (Chettri et al., 2015a). Anti-*Yersinia* LPS antibodies were used for coating that could not account for any lack of antibody response detected because whole cells were used first for coating and the results showed no lack of antibody response detected (data not show). Some studies suggested that coating of whole cells to microtiter plates can cause inconsistent results in ELISA (Poxton, 1995; Thirumalapura et al., 2006).

The agglutination results showed that only injection vaccination elicited agglutination titres and no agglutination was observed in any of the salmon vaccinated by immersion. This result was consistent with the results of serum antibody levels in the present study. In a previous study, bacterial agglutination was the highest in the serum of Atlantic salmon that were injected with Yersinivac-B and no agglutination was evident in the other salmon, including non-vaccinated fish (control), single dip vaccinated fish, bath vaccinated and orally vaccinated fish (Tonkes, 2010). Serological data and the results of the RPS in the immersion vaccines confirmed that there was no correlation between the level of protection against *Y. ruckeri* infection and the level of serum antibodies. In rainbow trout, majority of particles were remaining within skin and gill tissues for up to 24 days post-exposure demonstrated that local immune responses had an important role in the protection from pathogens following immersion immunisation (Moore et al., 1998). Ohtani et al. (2014) showed that *Y. ruckeri* bacterin was initially taken up via gill lamellae around 30 seconds following immersion immunisation in rainbow trout. The mechanism of protective immunity against *Y. ruckeri* should be further investigat-

ed to identify if this is due to a localised immune responses or a cell-mediated immune response.

Following the success of the ammonium sulphate inactivated vaccine from the challenge results, this study used the real-time PCR to determine gene expression of five genes of interest that were involved in the immune response of Atlantic salmon in immersion vaccination against yersiniosis. These genes were chosen from 17 genes which were used as surrogates to measure protection of a specific vaccine-induced biosignature that could be used to predict the vaccine success after vaccination but before the *Y. ruckeri* challenge (Bridle et al., 2012). These five genes were up-regulated in the fish after six weeks of vaccination with the ammonium sulphate inactivated vaccine. The up-regulated of hepcidin, LIM and actin-binding protein, and fish virus induced TRIM protein was consistent with the results of Bridle et al. (2012). Hepcidin was significantly up-regulated in expression post infection with *Y. ruckeri* in rainbow trout (Chettri et al., 2012). The results of this study suggest hepcidin has a role in the antibacterial defences of Atlantic salmon against yersiniosis (Bao et al., 2005; Hu, 2008). In contrast, myelin and lymphocyte protein and the bifunctional 3-phosphoadenosine 5-phosphosulfate synthetase showed the opposite results to those reported previously (Bridle et al., 2012). The different regulation of these genes may be due to different method of measurement. Our study confirmed three up-regulation genes (more than 2.5 fold change) may be used to predict efficacy of *Y. ruckeri* vaccine. It would be useful to continue investigate the other genes studied by Bridle et al. (2012) to increase our understanding of immune response genes as surrogate of protection in predict vaccine success.

In this study, *Y. ruckeri* cells were observed in the spleen of surviving fish three weeks after challenge. The spleen is a major secondary immune organ where plasma cells, lymphocytes and macrophages develop (Ellis, 1989) and is known as an important site of immune cell flux and bacterial replication during *Y. ruckeri* infection (Welch & Wiens, 2005; Wiens et al., 2006). A small number of positive fish with *Y. ruckeri* with a small number of *Y. ruckeri* cells present in three vaccinated groups compared to the non-vaccinated group (control). This may indicate that the vaccinated fish are protected

from the disease caused by *Y. ruckeri* by not immune to infection. Further studies are required to investigate the reinfection status of *Y. ruckeri* with the correlation of *Y. ruckeri* cells in fish organs after vaccination.

In conclusion, the present study has contributed to a better understanding of how different inactivation methods of *Y. ruckeri* influence vaccine efficacy in Atlantic salmon. Ammonium sulphate can be used to precipitate, preserve and purify protein. In this study, ammonium sulphate was successfully used for *Y. ruckeri* inactivation and demonstrates potential for bacteria inactivation for commercial fish vaccines. Using ammonium sulphate can overcome the disadvantages of cross-linking of proteins created during formalin inactivation and as a result produce more efficient vaccine for fish. Additionally, this study showed that there was no correlation between the level of serum humoral antibodies and the RPS in protecting the fish against *Y. ruckeri* infection. The prevalence of asymptomatic carriers was lower in vaccinated fish. The gene expression results contributed to our understanding of immune response genes as surrogate of protection in Atlantic salmon against *Y. ruckeri*.

CHAPTER 3.

EVALUATION OF HYPEROSMOTIC PRETREATMENT IN IMMERSION VACCINE OF ATLANTIC SALMON (*SALMO SALAR*) AGAINST *YERSINIA RUCKERI*

3.1 INTRODUCTION

Immersion vaccination is a natural route of antigen entry and has certain advantages over injection vaccination due to reduced stress on the fish, the convenience of vaccinating a large number of fish at one time and low labour costs (Moore et al., 1998; Nakanishi & Ototake, 1997). The most common forms of immersion vaccination used are direct immersion, hyperosmotic infiltration and spray. Hyperosmotic infiltration that immerses the fish in a hypertonic solution such as sodium chloride or urea for a short period of time followed by immersion in the vaccine, increased the vaccine uptake and enhanced the efficacy of several vaccines (Huising et al., 2003; Ototake & Nakanishi, 1992; Ototake et al., 1992). However, some authors have reported contradictory findings showing that hyperosmotic infiltration did not enhance antigen uptake or immune response (Anderson et al., 1979b; Tatner & Horne, 1983). Huising et al. (2003) showed that antigen-specific serum immune response was not detected following either hyperosmotic immersion or direct immersion but the mucosal antibody response following hyperosmotic immersion was stronger and lasted longer than that detected after direct immersion. In coho salmon (*Oncorhynchus kisutch*), serum agglutinins were induced five weeks after a hyperosmotic immersion treatment with either an inactivated suspension of *Aeromonas salmonicida*, *Vibrio anguillarum*, or a combination of the vaccines (Antipa & Amend, 1977). In contrast, Anderson et al. (1979b) found no specific antibody titres with either hyperosmotic immersion or direct immersion with *Y. ruckeri* bacterin in rainbow trout (*Salmo gairdneri*). Hyperosmotic infiltration may also cause stress and damage to the fish skin and gills therefore counteracting the potential improved efficacy afforded by the procedure (Nakanishi & Ototake, 1997).

Huising et al. (2003) showed that hyperosmotic immersion vaccination immediately increased the Na^+ and Cl^- concentration and osmolality in plasma but the homeostatic equilibrium was quickly restored when the fish was returned to fresh water. Laurent and Perry (1990) also reported a stress response in trout by the slight increase in plasma Na^+ and Cl^- level three hours after hyperosmotic immersion. A fundamental knowledge

about the effects of hyperosmotic pretreatment on vaccination with the effect on gills of Atlantic salmon is unclear.

Chloride cells in the gills are the primary site of ion absorption and secretion and play a pivotal role in adaptation to seawater and freshwater conditions (Katoh & Kaneko, 2003; Pritchard, 2003) It has been reported that changing salinity had affected the density of chloride and mucous cells (Laurent & Perry, 1991). Laurent and Hebibi (1989) reported that after transfer to artificial seawater, the density of filamental chloride cells was increased more than twofold in rainbow trout. In the smoltification process during which Atlantic salmon adapt to the changing water salinity from freshwater to seawater, the main physiological change is an increase in the number of chloride cells in the gills (Silverstone & Hammell, 2002). The chloride cells help to maintain osmoregulatory balance in hyperosmotic environments by pumping of sodium out of the blood. The proliferation of chloride cells in the gill epithelium involved in the elevation of plasma cortisol concentrations (Perry, 1997). Both the salinity increase or the infection resulted in increase of mucous cell number (Roberts & Powell, 2003). In sockeye salmon (*Oncorhynchus nerka*), the number of gill filament mucous cells was increased after transfer to seawater (Franklin, 1990). Hyperosmotic pretreatment may involve changing the number of chloride and mucous cells in the gills but no study has shown these effects of hyperosmotic immersion on fish gills.

Little is known about the effects of hyperosmotic infiltration on the efficacy of bacterin against yersiniosis in Atlantic salmon. This study has been undertaken to compare hyperosmotic infiltration with direct immersion in an ammonium sulphate inactivated *Y. ruckeri* whole-cell vaccine that has previously been used successfully to vaccinate Atlantic salmon (chapter 2 results). The serum antibody levels and the gill structure were also investigated in this current study. This study aimed to conclude that hyperosmotic pretreatment may provide protection-effective over direct immersion immunisation in Atlantic salmon against *Y. ruckeri*.

3.2 MATERIALS AND METHODS

3.2.1 Fish

Atlantic salmon (*Salmo salar*) weighing 2 g were provided by Salmon Enterprises of Tasmania (SALTAS) and were acclimatised to laboratory conditions for one week prior to the experiment. The fish were held in 1000 L tanks with a semi-recirculating fresh-water at 15 ^{o}C and were then moved to five 250 L net enclosures after vaccination. Fish were fed daily to satiation with a commercial diet (Skretting) and starved for 24 h prior to sampling. Fish kidneys were sampled from two or three fish per treatment and cultured on blood agar plates to confirm the absence of *Y. ruckeri* before vaccination. pH, ammonia and chlorine were checked daily to maintain water quality. This experiment was approved by the University of Tasmania Ethics Committee (Animal Ethics Number A0012285).

3.2.2 Preparation of bacterins

Formalin inactivated bacterin and ammonium sulphate inactivated bacterin were prepared as described in section 2.2.2.

3.2.3 Vaccination

Two hundred fish were used for each of five treatments which included ammonium sulphate inactivated bacterin, formalin inactivated bacterin, ammonium sulphate inactivated bacterin with hyperosmotic infiltration (all dip immersion), and positive and negative control groups which received formalin-inactivated bacterin by intraperitoneal injection or no vaccine respectively. For the vaccination, the fish were dipped in a 3 L bucket containing a 1:10 dilution of one of the three vaccines for a period of 60 s with constant aeration (dipping 50 fish/time). The fish vaccinated using hyperosmotic infiltration were dipped in a 4.5% NaCl solution for 3 min and then in ammonium sulphate inactivated bacterin for 60 s. After each vaccination the fish were dipped in freshwater to remove excess vaccine. The control fish were dipped in freshwater only

for 60 s (200 fish). The positive control fish (203 fish) were vaccinated by intraperitoneal injection (50 µL/fish) with the formalin inactivated bacterin.

3.2.4 Challenge

At 12 weeks post vaccination, fish (11 g) from each treatment (n=90) were challenged with *Y. ruckeri* (2.5×10^8 colony forming units (CFU)/ mL for 1 h) in air saturated 20 L buckets with fresh water at 15 ^{o}C. Fish were challenged and mortality was monitored as described in section 2.2.4. The experiment ended at 15 days after challenge and RPS was used to evaluate vaccine efficacy.

3.2.5 Sampling

From each treatment, 10 fish were sampled before vaccination (time 0) and at 12 weeks post-vaccination. Due to the small size of the fish, blood was only collected at week 12 post-vaccination. Sampled fish were anaesthetised with clove oil (1 mL in 1 L of water) prior to blood collection from the caudal vein using a 0.3 mL syringe and a 31 gauge needle, and placed into a 0.6 mL eppendorf tube. Blood samples were allowed to clot overnight at 4 ^{o}C. Following centrifugation at 500 x *g* for 10 min at 4 ^{o}C serum was recovered and stored at -20 ^{o}C. In addition, gills from five fish from each treatment were sampled after vaccination (week 0). These gills were fixed in Davidson's fixative (3 parts 95% ethanol, 2 parts 37-40% formaldehyde, 1 part glacial acetic acid and 3 parts filtered sea water) for at least 48 h before being transferred to 70% ethanol for storage and later histological examination.

3.2.6 Blood analysis

3.2.6.1 Serum ELISA

ELISA for detection the presence of specific anti-Yersinia antibodies in the blood serum were performed as described in section 2.2.6.1.

3.2.6.2 Bacterial agglutination

Due to the limited volume of blood obtainable from less than 10 g fish, pools of serum were used for the agglutination assay. Two pools of five fish from each treatment were used for analysing agglutination titres. Agglutination titres against *Y. ruckeri* in fish serum were determined as described in section 2.2.6.2.

3.2.7 Histology

Davidson's fixed gills were transferred to 70% ethanol until routinely processed for histology. This processing included dehydration in a graded ethanol bath (80% – 100%), clearing in xylene, paraffin-embedding, sectioning at 5 μm on microtome (Microm, Heidelberg, Germany), and then staining with haematoxylin and eosin using an automatic staining machine (Shandon, Linistain GLX, Waltham, USA). Finally, these sections were mounted on glass slides with distrene–plasticiser–xylene (DPX). These sections were then examined for mucous cells and chloride cells by using light microscope at magnifications of 40 – 400 X. Mucous cell and chloride cell counts were carried out on 5 fish for each treatment excluding the injection group (400 X magnification). Five well-orientated filaments were randomly selected and 4 inter-lamellar units (ILUs) from each filament were randomly chosen (total 20 inter-lamellar units per fish). The number of mucous cells and chloride cells present on each ILU were counted and the results averaged for each treatment.

3.2.8 Statistical analysis

Survival was analysed by using SigmaPlot 11.0, Kaplan-Meier Survival Analysis, with the multiple comparisons performed using the Holm-Sidak method including adjustment of the P value to avoid type I errors. The analysis of results from serum antibody levels, mucous cells and chloride cells were performed by using a one-way ANOVA. It was also used to compare differences between treatments as appropriate, and if significant followed by a Turkey's post hoc test to identify where significant differences occurred among treatment means. The results were considered significant when $P <$

0.05. Data input and statistical analysis were achieved using SPSS 22.0 software (SPSS Inc., USA).

3.3 RESULTS

Fish mortalities started at day 4 post challenge. The negative control fish had a lower survival rate (21.1%) than the fish vaccinated with ammonium sulphate inactivated bacterin (35.6%), formalin inactivated bacterin (35.6%), ammonium sulphate inactivated bacterin with hyperosmotic infiltration (46.7%) and the positive control (97.8%) (Figure 3.1). However, excluding the i.p. injected positive control, the ammonium sulphate inactivated bacterin with hyperosmotic infiltration was the only treatment shown to be significantly different from the negative control (unvaccinated fish) (P = 0.008).

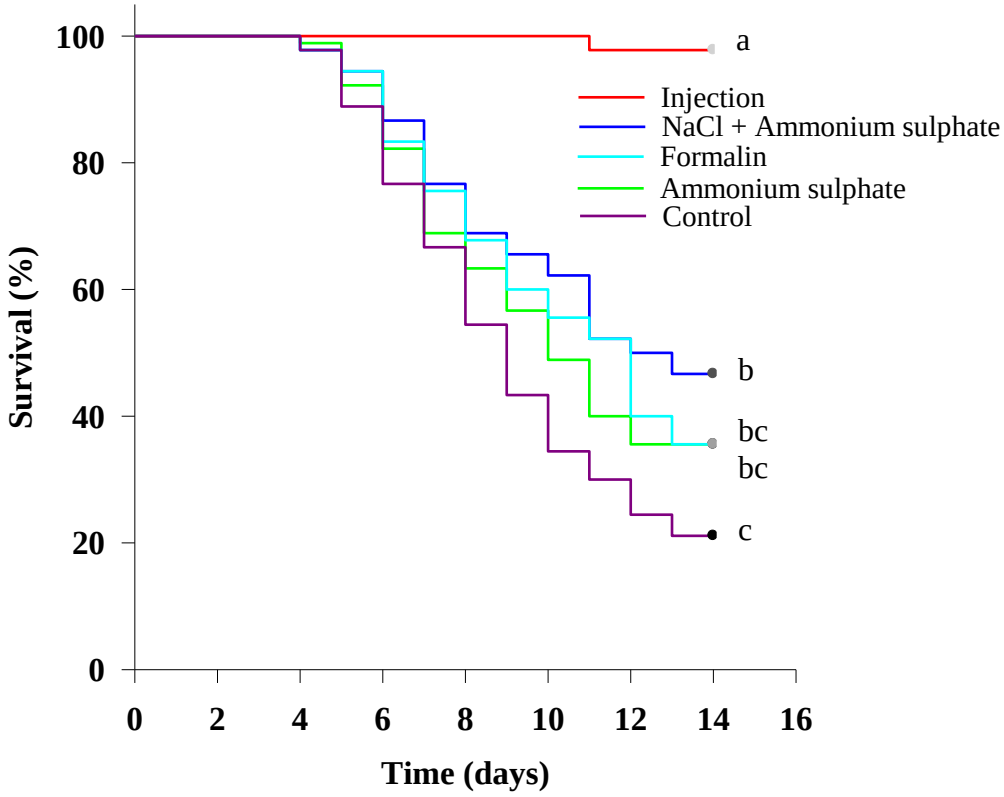

Figure 3.1 Effects of immersion vaccination using different bacterin preparations (formalin-inactivated, ammonium sulphate inactivation, ammonium sulphate inactivated bacterin with hyperosmotic infiltration) on survival of Atlantic salmon after disease

challenge. Twelve weeks post-vaccination, five groups of 90 Atlantic salmons each were challenged by immersion with 2.5×10^8 CFU/mL of *Y. ruckeri*. Different letters indicate significant differences (Survival analysis, SigmaPlot 11.0, $P < 0.05$)

The mortality in vaccinated fish varied from 53.3% to 64.4%, giving the RPS of the ammonium sulphate vaccinated fish with hyperosmotic infiltration 32.4%, ammonium sulphate vaccinated fish 18.3% and formalin vaccinated fish 18.3% (Table 3.1).

Table 3.1 Protection of Atlantic salmon following vaccination using bacterin produced by different inactivation methods and hyperosmotic treatment methods

Treatments	**Number of fish**	**Specific mortality**	**Survival** (%)	**Mortality** (%)	**RPS** (%)
Ammonium sulphate	90	58	35.6	64.4	18.3
NaCl + Ammonium sulphate	90	48	46.7	53.3	32.4
Formalin	90	58	35.6	64.4	18.3
Injection	90	2	97.8	2.2	97.2
Control	90	71	21.1	78.9	0

There was no serum antibody titre at week 12 in any of the immersion vaccinated groups or negative control fish.

Bacterial agglutination was evident at a titre ranging from 4 to 8 only in the positive control fish (injection vaccination). No agglutination was seen in the unvaccinated fish serum (control), the ammonium sulphate vaccinated fish serum, ammonium sulphate vaccinated fish serum with hyperosmotic infiltration and formalin vaccinated fish serum.

The means of chloride cell counts (Figure 3.2) were significantly different between treatments ($P < 0.001$). Post hoc tests revealed that chloride cell counts were significantly higher in formalin vaccinated fish compared to that of unvaccinated fish (3.88 ± 0.03, 3.26 ± 0.09 chloride cells ILU^{-1}, respectively, $P < 0.001$). Chloride cell counts in

unvaccinated fish (control) (Figure 3.2) were not significantly higher compared to ammonium sulphate vaccinated fish with or without hyperosmotic infiltration ($P > 0.05$).

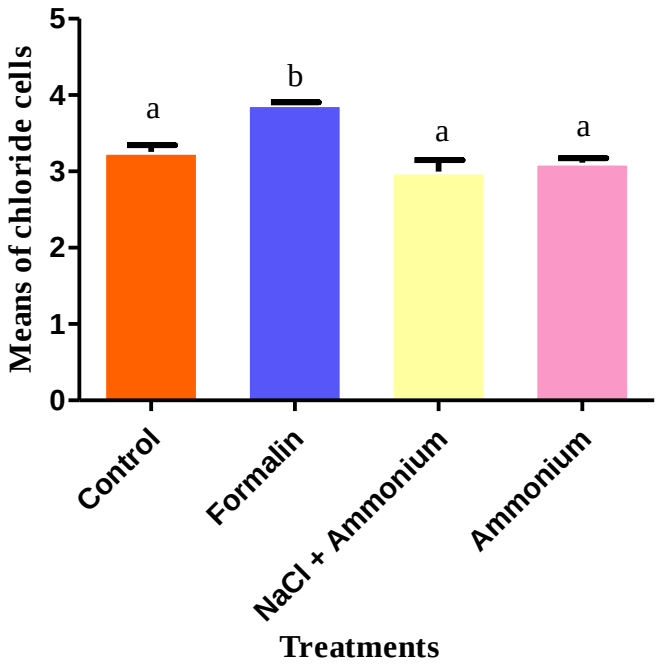

Figure 3.2 Chloride cell counts per interlamellar unit (ILU) for Atlantic salmon (mean ± S.E.)

The mean of mucous cell counts (Figure 3.3) were significantly different between treatments ($P < 0.001$). Post hoc tests revealed that mucous cell counts were significantly higher in ammonium sulphate vaccinated fish with hyperosmotic infiltration (1.10 ± 0.09 mucous cells ILU^{-1}) compared to that of unvaccinated fish (0.57 ± 0.04 mucous cells ILU^{-1}, $P < 0.001$). Mucous cell counts in formalin vaccinated fish (0.73 ± 0.03 mucous cells ILU^{-1}) and ammonium sulphate vaccinated fish without hyperosmotic infiltration (0.52 ± 0.04 mucous cells ILU^{-1}) were not significantly different compared to that of the unvaccinated fish ($P > 0.05$).

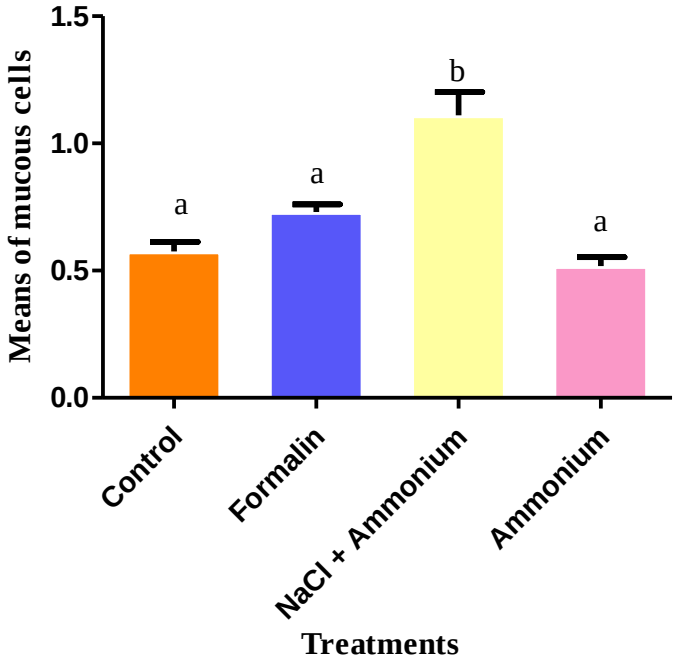

Figure 3.3 Mucous cell counts per interlamellar unit (ILU) for Atlantic salmon (mean ± S.E.)

3.4 DISCUSSION

The survival rate of the fish vaccinated with the ammonium sulphate inactivated bacterin with hyperosmotic infiltration was greater by 25.6% and significantly different than the survival rate for unvaccinated control fish. Vaccination with the other bacterin preparations resulted in no significant difference in survival post challenge to the unvaccinated control group except for the group injected with the bacterin which had survival which was significantly higher than all other groups. These results strongly suggest that hyperosmotic infiltration can improve protection afforded by immersion vaccine against *Y. ruckeri* for Atlantic salmon. Previous research has shown that hyperosmotic infiltration, where vaccinated common carp (*Cyprinus carpio*) were immersed in 4.5% NaCl for two minutes before vaccine administration, enhanced the vaccine uptake or vaccine efficacy (Huising et al., 2003). Increased antigen uptake was shown for rainbow trout (*Oncorhynchus mykiss*) (see Fender & Amend, 1978), channel catfish (*Ictalurus punctatus)* (see Thune & Plumb, 1984) and increased vaccine protection in

sockeye salmon (*Oncorhynchus nerka*) (see Antipa et al., 1980). Amend and Fender (1976) suggested that hyperosmotic infiltration had a hydrating effect on membranes of organs such as gills or the lateral line canal and it allows the antigen to infiltrate into fish more easily. Therefore, Atlantic salmon in the hyperosmotic infiltration group in this study may have taken up more *Y. ruckeri* bacterin compared to the non-hyperosmotic infiltration group which resulted in higher protection. Ototake et al. (1996) had quantified antigen uptake in hyperosmotic infiltration (HI) and direct immersion (DI) by using labelled bovine serum albumin (BSA) in rainbow trout. The results showed that BSA concentrations in HI group were significantly higher than those of the DI group for the tissues of skin, gills and intestine. This method may apply for further studies to quantified *Y. ruckeri* uptake in hyperosmotic pretreatment and direct immersion. The relative percent survival (RPS) of three modes of vaccine application in this study did not meet the suggested 60% for effective protection (Amend, 1981), possibly due to the high challenge dose (2.5×10^8 CFU/mL). The size of the fish used in this study was smaller than the fish used in Chapter 2. They were 2 g at vaccination and were challenged 12 weeks later with the fish size around 11 g. Therefore, it seems that the fish size would have been contributed to the high mortality after challenge of three immersion immunisation groups. Furthermore, a study by Brudeseth et al. (2013) showed that fish of less than 2 g weight have a poorly developed immune competency and this may be another reason for the poor vaccine efficacy.

Gills are the major route of antigen entry in bath immunisation (Bowers & Alexander, 1981; Kawahara & Kusuda, 1988; Ohtani et al., 2014). This study has shown that the gills of ammonium sulphate vaccinated fish with hyperosmotic infiltration did not contain different number of chloride cells, but showed significantly higher numbers of mucous cells compared to unvaccinated fish. Chloride cells perform the roles of acid-base balance, ionic regulation and gas transfer (Perry, 1997). Perry (1997) also showed that the proliferation of the chloride cells is beneficial to ionic regulation, however, it impedes respiratory gas transfer by causing a thickening of the blood-to-water diffusion barrier. There was not a significant difference in the number of chloride cells in the hyperosmotic infiltration group compared to control group in this study. This suggests

that the hyperosmotic pretreatment had no adverse effects on the fish. Mucous cells contain polyanions, mucins composed of glycoproteins that can prevent toxicant entry into the gill epithelium by trapping toxicants (Perry & Laurent, 1993). Despite being beneficial in reducing toxicant entry, the consequence of mucous cell proliferation may be an increase in the distance for gas exchange along the secondary lamellae and causing hypoxic conditions (Ultsch & Gros, 1979). The number of mucous cells in fish gill of the hyperosmotic infiltration group was significantly higher than in the gills of fish from the unvaccinated group. However, as the hyperosmotic pretreatment is short in duration (3 minutes), the fish may recover when they get back to their normal water environment. Further studies with more replication at different time points are needed to determine the effects of hyperosmotic infiltration on the chloride cells and mucous cells.

Before *Y. ruckeri* challenge (week 12 post vaccination), no antibodies against *Y. ruckeri* could be detected in the three groups: the immersion vaccinated fish, the positive control fish (injection vaccination) and the negative control fish (unvaccinated fish) using ELISA. The present results were consistent with a recent research has shown that serum antibody levels reacting with *Y. ruckeri* in rainbow trout before the challenge were not significantly different in injection vaccinated fish, immersion vaccinated fish, and unvaccinated control fish (Chettri et al., 2013). There was no antibody titre in injection vaccinated fish when the challenge results showed an RPS of 97.2%. There was no correlation between the level of serum antibody production and the level of protection afforded by immersion vaccine.

The agglutination results at 12 weeks post vaccination showed that injection vaccination elicited an adaptive immune response. However, agglutination was not observed in any of the salmon vaccinated by immersion. While this does not mean a lack of an adaptive immune response in these fish, when combined with the low titres in the i.p. injected fish, it does suggest that the immune system of 2 g Atlantic salmon may not be fully developed. It is also possible that agglutination antibody titres may have been produced and measurable prior to 12 weeks post vaccination. Therefore, measurement of antibody titres at earlier time points in post vaccination should be included in future research.

Furthermore, measurable serum agglutination or serum anti *Y. ruckeri* antibodies in immersion vaccinated Atlantic salmon was not necessary for protection (Tonkes, 2010).

In conclusion, the present study has demonstrated that hyperosmotic infiltration can improve protection of a vaccine against *Y. ruckeri* for Atlantic salmon. It confirmed that ammonium sulphate can be used for *Y. ruckeri* inactivation and has the potential to be used to inactivate bacteria for other bacterin-based immersion vaccines.

CHAPTER 4.

EFFECTS OF SINGLE DIP AND DOUBLE DIP VACCINE APPLICATION IN EARLY LIFE STAGES OF ATLANTIC SALMON (*SALMO SALAR*) AGAINST *YERSINIA RUCKERI*

4.1 INTRODUCTION

Y. ruckeri can affect fish of all ages, but is most acute in salmonids during their hatchery stage. Yersiniosis may appear in Atlantic salmon smolt, particularly 3 - 6 weeks post transfer to marine grow-out sites and the number of fish affected is typically between 0.1 and 0.75% per week (Carson & Wilson, 2009). Clinical signs of yersiniosis in juvenile salmonid fish include changed behaviour, with fish moving sluggishly or swimming near the surface of water and an abnormal increase in mortality. Atlantic salmon with yersiniosis may also have darkening of the body. Other clinical signs of yersiniosis include unilateral or bilateral exophthalmos, haemorrhagic blood spots on the iris of the eye, haemorrhage with reddening along the lateral line, at the base of the pectoral and pelvic fins and on the head region. Enteric red mouth disease in rainbow trout is caused by *Y. ruckeri* infection and is characterised by subcutaneous haemorrhage in the throat and mouth (Carson & Wilson, 2009; Tobback et al., 2007).

The first commercial ERM vaccine was produced and licensed in 1976 as formalin-killed whole cells of *Y. ruckeri* (see Bridle et al., 2012). In Tasmania, Atlantic salmon are vaccinated once by bath immersion at 5 g. After a study (Costa et al., 2011) which found that vaccination using the dip form of immersion resulted in a higher level of protection compared to bath vaccinations, the Tasmanian salmonid industry converted to use more dip vaccinations. Fish are usually vaccinated when they weigh 1-3 g and then again at 5 g, as a booster. The ontogeny of immune system in salmonids has been studied to identify protective immune mechanism in early life stages and the ways that they cope with the pathogens in the natural aquatic environment. In rainbow trout, carps and zebrafish, the maturation of humoral immune responses is delayed until one or two months (Zapata et al., 2006). No indication in the gene expression of TCR-α, RAG-1 and IgM (BCR) of 0.15 g Atlantic salmon fry indicated they had undeveloped immune response (Gadan et al., 2013). Johnson et al. (1982) suggested that only fish of about 2.5 g can achieve long term protection post vaccination and fish less than 1 g had a poor response to vaccination because of a poorly developed immune response. However, mortality still occurs at hatcheries and it appears that yersiniosis affects fish from very early stages of development. The ability to vaccinate fish and protect them against *Y.*

ruckeri as early as possible would be an obvious advantage. Thus vaccine application strategies in early life stages of Atlantic salmon need further investigation. The aim of this study was to investigate the vaccine performance of immersion vaccination and compare the effectiveness of single dip and double dip vaccination of Atlantic salmon fry. The vaccination experiment was performed at 0.13 g fish when they were started commencement of exogenous feeding. If this vaccine performance is effective then it will protect fish against *Y. ruckeri* in their early life stages.

4.2 MATERIALS AND METHODS

4.2.1 Fish

The Atlantic salmon (*Salmo salar*) alevin used in the experiment weighed approximately 0.13 g and were provided by Lonnavale hatchery. The fish were acclimated for three days to laboratory conditions prior to the experiment. Fish were held in 30 L mesh enclosure inside 1000 L holding tanks of a UV-sterilized freshwater recirculating system at 11 ^{o}C. The commencement of exogenous feeding of all fish obtained from the hatchery did not begin until three days after arrival at the laboratory of the university. The fish were fed with a commercial crumble feed (Atlantic salmon starter crumbles, Skretting, Cambridge, Tasmania). Fish were fed every hour from 9 am to 5 pm until visually observed satiation occurred. Water quality, including pH (6.8-7.2), ammonia (<0.5 mg L^{-1}), nitrite (<0.05 mg L^{-1}), nitrate (<5 mg L^{-1}) and chlorine (<0.01 mg L^{-1}) was checked daily. This experiment was approved by the University of Tasmania Ethics Committee (Animal Ethics Number A0012285).

4.2.2 Preparation of bacterin

Frozen *Y. ruckeri* stock, strain UTYR001A, was obtained from the Institute for Marine and Antarctic Studies (IMAS), UTAS culture collection, and used to prepare the bacterin and challenge inoculation. A 250 mL brain heart infusion (Oxoid, England) starter was inoculated and shaken for 20 h at room temperature (18˚C), then added to a 10 L Nalgene container with 7 L brain heart infusion media and cultured with constant aeration for 30 h at room temperature (18˚C). The number of bacterial cells was counted

using a Neubauer hemocytometer after inactivating the cells with 10% formalin. The formalin was used to inactivate bacteria by adding formalin to 1.2×10^{10} cells/mL to achieve a 0.3% final concentration. The number of colony forming units (CFU) per mL bacterins used for inactivation was quantified by triplicate plating of the bacteria on LB agar (Sigma, USA) plates with a ten-fold dilution series. After the inactivation, the vaccine was left for 28 h at room temperature before it was inoculated on LB agar plates with 100 µL of inactivated bacteria in triplicate to check the viability. The absence of bacterial growth indicated the non-viability after 24 h. The bacterin was stored at 4 ^{o}C until used for immunisation.

4.2.3 Vaccination

Around four hundred fish were used for each treatment. Feed was withheld from fish for 24 h before they were administered an immunisation via immersion. Vaccinated fish were divided into two treatments. One group of fish was vaccinated twice: the first time prior to first feed (0.13 g) for the first dip and then at an average weight of 1 g for the second dip. Another group was only vaccinated once when the average weight was 1 g. During vaccination, the fish were dipped in a 3 L bucket containing a 1:10 dilution of the vaccine for 60 s (dipping 50 fish/time) under constant aeration. After vaccination, the fish were dipped in freshwater to remove excess vaccine before being returned to their respective tanks. The control fish were dipped in freshwater only for 60 s (400 fish). Feeding was resumed 24 h after booster immunisation with commercial feed.

4.2.4 Challenge

At the average weight of 5 g post vaccination, fish from each treatment (n=60) were randomly selected and challenged with *Y. ruckeri* (2.5×10^{7} colony forming units (CFU)/mL for 1 h) in air saturated 20 L buckets with fresh water at 12^{o}C. Fish were challenged and mortality was monitored as described in section 2.2.4. RPS was used to evaluate vaccine efficacy at the end of the experiment (30 days after challenge).

4.2.5 Sampling

To demonstrate *Y. ruckeri*-free status of the population used for this experiment, 10 fish (approximately 0.13 g body weight) were lethally anaesthetised with clove oil (0.3 ml in 1 L water). Each fish was briefly rinsed to remove traces of anaesthetic and homogenised in 1 mL PBS. This homogenate was incubated on TSA plates for 24 h at 18°C. The colonies were harvested from TSA plates and analysed using PCR to confirm *Y. ruckeri*-free status.

An additional 10 fish from each treatment were sampled before first vaccination before first feed, before second vaccination when the average weight of fish was 1 g, and before challenge when the average weight of fish was 5 g. Due to the small size of the fish, blood was only collected when the fish reached the average weight of 5 g. Sampled fish were anaesthetised with clove oil (0.3 mL in 1 L of water) prior to blood collection. Blood was collected from the caudal vein using a 0.3 mL syringe and a 31 gauge needle, and then placed into a 0.6 mL eppendorf tube. Blood samples were allowed to clot overnight at 4 ^{o}C. Serum was recovered by centrifuging at 500 x *g* for 10 min at 4 ^{o}C and stored at -20 ^{o}C. Gills, intestine and spleen were also collected and placed in a 5 mL tube of RNA preservation solution (25 mM Sodium Citrate, 10 mM EDTA, 70 g ammonium sulphate/100 mL solution, pH 5.2). 10 small fry at first feed and at the average weight of 1 g were collected whole from each treatment. These samples were refrigerated overnight at 4 ^{o}C and then stored at -20 ^{o}C.

4.2.6 Serum ELISA

ELISA for detection the presence of specific anti-*Yersinia* antibodies in the blood serum was performed as described in section 2.2.6.1.

4.2.7 Quantitative Real-time PCR Analysis (mRNA expression of RAG-1, IgM, and TCR-α)

Immunocompetence was assessed at week 21 using 30 fish (~ 5.0 g/fish, 6 fish/treatment) including unvaccinated fish (control) and two vaccinated groups; includ-

ing single dip vaccinated fish and double dip vaccinated fish. mRNA expression of RAG-1, IgM, and TCR-α was determined with different treatments and real-time PCR was carried out on spleen samples.

4.2.7.1 RNA extraction, DNA decontamination and reverse transcription

Total RNA was extracted from spleen samples. Approximately 2 mg of spleen were cut into very small pieces and transferred to a 2 mL round bottom tube with 100 µL of RNA extraction buffer [5 M guanidine isothiocyanate, 1% Triton X 100, 50 mM Tris (pH 7)]. Then, 100 µL of isopropanol with pink-pellet paint co-precipitate (1:500 Pink: Iso) was added and precipitated by centrifugation at 16,000 x *g* for 10 min at RT. The supernatant was discarded and the pellet was incubated for 10 min at 37 ºC in 200 µL of extraction buffer (4 M Urea, 1% SDS, 0.2 M sodium chloride, 1 mM Sodium citrate) supplement with 5 µL of proteinase K (20 mg/mL; Bioline Australia) with occasional mixing by vortex to resuspend the pellet. Protein, cellular debris, and detergent were then removed by precipitation with the addition of 100µL of 7.5 M ammonium acetate centrifugation at 14,000 x *g* for 10 min 18 ºC. Nucleic acids were then precipitated from the supernatant by adding one volume of isopropanol with pink-pellet paint co-precipitate (1:500 Pink:Iso) and centrifugation at 16,000 x *g* for 10 min at RT. The nucleic acid pellet was then washed twice with 75% ethanol and resuspended in 180 µL of molecular grade water and 20 µL of 10 X DNAse buffer at 37 ºC for 5-10 min. RNA was reversely transcribes using a 50 µM of Oligo dT18 primer mix [1 µL of 10 mM dNTP, 2 µL of 10 X RT buffer, 0.25 µL of RNAse inhibitor, and 0.25 µL of reverse transcriptase (M-MuLV-RT)]. Reactions were performed in a gradient cycler (Mastercycler Gradient, Eppendorf, Germany) with 20.5 µL reaction volumes containing 500 ng of total RNA extraction. The reactions were run under the following conditions: 65 ºC for 10 min, 42 ºC for 50 min, 70 ºC for 15 min and 12 ºC indefinitely.

4.2.7.2 cDNA Template

Following cDNA generation, the samples were diluted 5 folds with water by adding 80 µL of molecular grade water to each sample. Five standards were then created by

pooling 5 µL of each sample into a single eppendorf tube to create standard 1. The other four standards were made by serially diluting in 5-fold dilution with water (i.e. 40 µL into 160 µL of water, the highest to the lowest). The negative sample was made by pooling 2 µL of RNA of 8 samples of 8 different groups and diluted at the same concentration as the samples for cDNA preparation (in 20 folds). All samples, standards, negative sample and non-template control (water) were then transferred to the cDNA plate and held at 4 ^{o}C for later use.

4.2.7.6 Real-Time qPCR for Gene Expression Analysis

A Real-Time qPCR run was performed in duplicate and each Real-Time qPCR master mix contained 2.92 µL of water, 0.04 µL of 100 mM F primer, 0.04 µL of 100 mM R primer, 5 µL of 2 X My Taq HS Mix (Bioline) and 2 µL of template. These were then transferred to a qPCR plate (10 µL/well) of the iQ5 machine by using a multichannel pipette. A film seal was applied to the plate and the plate was then short spun at 500 x *g* at 4 ^{o}C. A run protocol and the plate setup were selected using real-time software. A generic protocol for SensiFast was: 94 ^{o}C for 2 min, then 95 ^{o}C for 5 sec, 55 ^{o}C for 20 sec and 72 ^{o}C for 10 sec for 40 times and then for melting curve 95 ^{o}C for 1 min, 55 ^{o}C for 1 min and 55 ^{o}C+1 ^{o}C/10 sec for 36 times, until 90 ^{o}C. Primers used for real-time qPCR are shown in Table 4.1. The amplification efficiency of the standards should be 85-105% with an R^2 higher than 0.98. This real-time qPCR was used to express the three different genes of RAG-1, IgM, and TCR-α.

Table 4.1 Primers used for real-time qPCR

Gene	Name	Direction	Sequence (5'-3')
IgM heavy chain membrane bound form IgM	IgM	Forward	TCT GGG TTG CAT TGC CAC TG
		Reverse	GTA GCT TCC ACT GGT TTG GAC
Recombination activating gene-1	RAG-1	Forward	CCT AAC ACC TCT AGG CTT GAC
		Reverse	GCT TCC CTG TTT ACT CGC
T cell receptor alpha chain	TCR-α	Forward	GCC TGG CTA CAG ATT TCA GC
		Reverse	GGC AAC CTG GCT GTA GTA AGC
Elongation factor 1a	EF1a	Forward	TGATTGTGCTGTGCTTA
		Reverse	AACGCTTCTGGCTGTAGG
Beta actin	β-actin	Forward	TTGCGGTATCCACGAGAC
		Reverse	TAGAGGGAGCCAGAGAGG

4.2.8 Statistical analysis

Survival was analysed using SigmaPlot 11.0, Kaplan-Meier Survival Analysis. Multiple comparisons were performed using the Holm-Sidak method including adjustment of the P value to avoid type I errors. The results were considered significant when $P < 0.05$. Data input and statistical analysis were achieved using SPSS 22.0 software (SPSS Inc., USA). mRNA expression levels were determined by qBase Plus software (Biogazelle, Belgium) using the mean expressions of two reference genes – elongation factor 1a (EF1a) and b-actin. qBase Plus software was also used to analyse the qPCR data including statistical analysis (ANOVA) for significant difference in the mean of expression genes of each group. The data were tested using a log-normal distribution in ANOVA.

4.3 RESULTS

Survival of unvaccinated fish (the negative control fish) was not significantly different from either single dip vaccinated fish or double dip vaccinated fish ($P > 0.05$). Fish mortalities started at day 4 post challenge. The fish vaccinated at 1 g (single dip) had a

survival rate of 28.3% and the fish vaccinated before first feed and again at 1 g (double dip) had survival rate of 25.0% whereas the negative control fish had survival rate of 10% by 30 days post challenge (Figure 4.1).

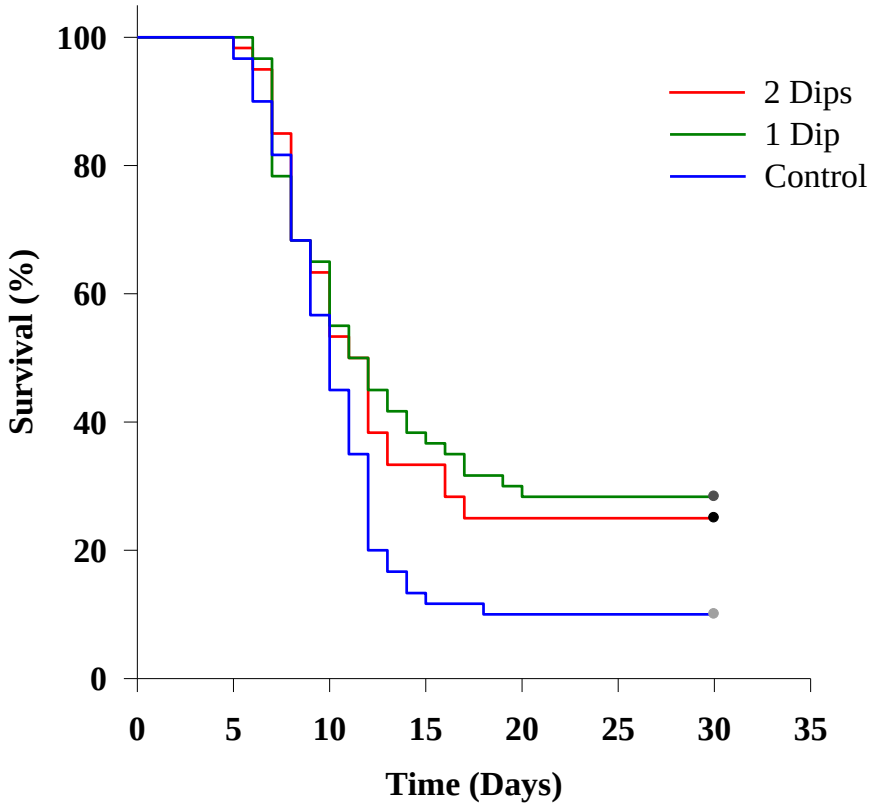

Figure 4.1 Effects of vaccination (single dip or double dip vaccination) on survival of Atlantic salmon. At the average weight of 5 g post vaccination (21 weeks post-vaccination), three groups of 90 Atlantic salmon each were challenged by immersion with 2.5×10^7 CFU/mL of *Y. ruckeri*. There were no significant differences in survival between any vaccinated groups and the unvaccinated controls (Survival analysis, SigmaPlot 11.0, $P > 0.05$)

The mortality in vaccinated fish varied from 71.7% to 75.0%, giving the RPS of the single dip vaccinated fish and double dip vaccinated fish at 20.4% for 1 Dip and 16.7% for 2 Dips (Table 4.2).

Table 4.2 Protection of Atlantic salmon following vaccination using single dip immersion only or with a single dip immersion follwed by a booster dip immersion

Treatments	**No. of fish**	**Specific mortality**	**Survival** (%)	**Mortality** (%)	**RPS** (%)
2 Dips	60	45	25.0	75.0	16.7
1 Dip	60	43	28.3	71.7	20.4
Control	60	54	10.0	90.0	0.0

No antibody titre was detected in the unvaccinated fish (control), single dip vaccinated fish and double dip vaccinated fish.

The immune status of the two different groups of vaccinated fish at the average weight of 5 g was examined by analysing the mRNA expression of IgM, RAG-1 and TCR-α by real-time PCR. The mean expression levels of IgM in control fish (unvaccinated fish) was not significantly different from the other two vaccinated groups, including single dip vaccinated fish and double dip vaccinated fish ($P > 0.05$) (Figure 4.2). However, the mRNA expression of RAG-1 and TCR-α of all treatment groups including the control was below the limit of detection.

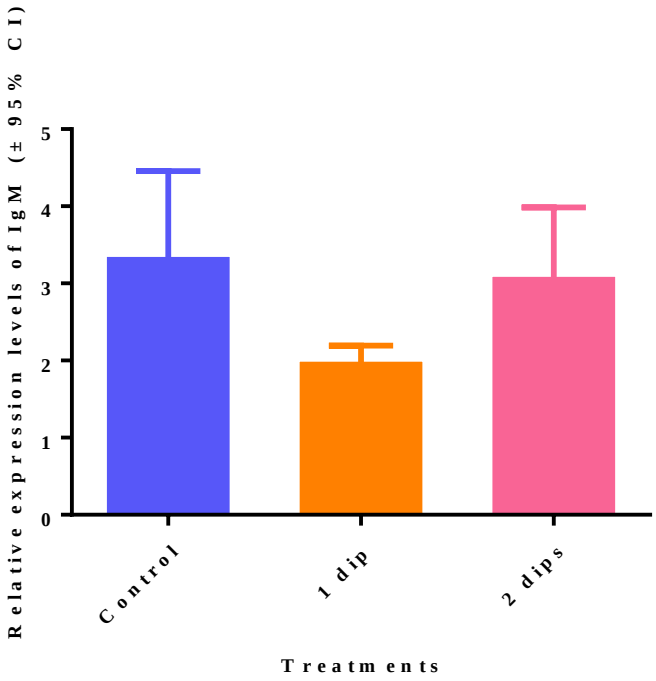

Figure 4.2 The mRNA expression of IgM at 5 g fish of Atlantic salmon, showing no significant difference in expression levels between groups ($p>0.05$), n = 6, One-way ANOVA.

4.4 DISCUSSION

The survival of Atlantic salmon in either single dip vaccinated fish or double dip vaccinated fish was not significantly different from unvaccinated fish (negative control fish) after 30 days of *Y. ruckeri* challenge ($P > 0.05$). This may be due to the high challenge dose (2.5×10^7 CFU/mL). A previous study showed that the survival of a double dip vaccinated group was significantly higher than single dip or bath vaccinated groups in immersion vaccination of *Y. ruckeri* against ERM in rainbow trout (Chettri et al., 2015a). However, the results of this study did not show any significant difference between the survivals of salmon exposed to a single dip immersion or a single dip with a booster (double dip).

There was no significant difference in expression levels of IgM between the unvaccinated fish (control) and the other two groups of vaccinated fish. The mRNA expression of

RAG-1 and TCR-α at the time of challenge (5 g) of all treatments exhibited no indication of expression levels. This may indicate that Atlantic salmon fry that are 1 g or smaller are not immunocompetent. This is consistent with previous studies that showed Atlantic salmon fry below 1 g had undeveloped immune response as there was no indication of TCR-α, RAG-1 and IgM (BCR) in the gene expression of 0.15 g fish (Gadan et al., 2013). Furthermore, Johnson et al. (1982) suggested that fish less than 1 g had a poor response to vaccination because of a poorly developed immune response, and only fish of about 2.5 g can achieve long term protection post vaccination. Most likely the first vaccination at 0.13 g and the second vaccination at around 1 g were given when the fish were not immunocompetent, and this may explain the lack of difference in survival rates.

Serum antibodies against *Y. ruckeri* were not found in either of the two vaccinated groups, or the unvaccinated group at 12 week post immersion vaccination. This may indicate that the humoral immunity was not induced by immersion vaccination. A low level to undetectable level of serum antibody titres was reported after immersion vaccination in rainbow trout, sockeye salmon, carp (Baba et al., 1988; Croy & Amend, 1977; Liewes et al., 1982; Sakai et al., 1984; Tatner & Horne, 1986). Serum antibody levels in rainbow trout immunized by immersion vaccination were induced after 14 to 30 days post *Y. ruckeri* challenge (Chettri et al., 2015a). In rainbow trout, *Y. ruckeri* specific antibody levels of vaccinated groups were significantly increased compared to unvaccinated group 16 days post *Y. ruckeri* challenge, but all the fish showed low antibody reactivity and an insignificant difference in pre-challenge vaccinated and unvaccinated fish (Chettri et al., 2015a). These studies indicated that *Y. ruckeri* specific antibody levels were only induced after *Y. ruckeri* challenge. This may be a reason why no serum antibodies were found in the present study. The production of *Y. ruckeri* specific antibody levels in pre-challenge and post challenge fish should be investigated to better understand the role of humoral immunity in immersion vaccination.

In conclusion, this study has contributed to a better understanding the vaccine performance of single dip and double dip immersion vaccination of small hatchery-sized fish.

When Atlantic salmon fry weighed around 1 g, the vaccine did not work even as double dip. This appeared to be due to the undeveloped immune response in fish this size. The lack of measurable serum antibodies against *Y. ruckeri* indicates the humoral immunity was not induced by immersion vaccination.

CHAPTER 5.

GENERAL DISCUSSION

This thesis focused on the effectiveness of different administration methods of immersion immunisation of Atlantic salmon to improve vaccine efficacy against yersiniosis. Vaccine against yersiniosis was prepared by three different methods of inactivating *Y. ruckeri* including a novel method using ammonium sulphate that has not been used previously for inactivation of bacteria for fish. Ammonium sulphate is the most commonly used salt for protein purification and can preserve potential antigens and nucleases. Using ammonium sulphate to inactivate *Y. ruckeri* to produce vaccines may be overcome the disadvantage of conventional formalin inactivated bacterin due to the lack of cross-linking of proteins. The successful use of ammonium sulphate is described in Chapter 2. This bacterin was then combined with hyperosmotic pretreatment in immersion vaccination. Chapter 3 demonstrated the improvement in protection of the fish from the hyperosmotic pretreatment group compared to the direct immersion group. Yersiniosis affects very young fish. A single dip vaccination at first feeding, 0.13 g mean weight and double dip immersion vaccination with the first dip applied at the first feeding were compared to determine which vaccination regime was more efficient. Unfortunately, vaccine efficacy was not achieved for either of the groups (Chapter 4).

5.1 DIFFERENT WAYS TO MEASURE VACCINE SUCCESS

Vaccine efficacy is normally evaluated by using survival rate after challenge with live pathogen (Munang'andu et al., 2014). Using challenge for testing vaccines has raised concerns about animal welfare when large numbers of fish were involved (Stokes et al., 2011) and the experiments may take long time depending when the protection is measured post vaccination. Measuring protective antibodies was recommended as a way to replace challenge experiments and the use of fish (Stokes et al., 2011). Traditionally, antibody levels are used to access vaccine efficacy as their common correlates of protection provide by a vaccine (Munang'andu et al., 2014; Plotkin, 2008). In mammals, many vaccines are licensed based upon set up antibody titres that act as correlates of protection (Munang'andu et al., 2014). However, antibodies associated with protection have been observed in fish following immersion vaccination only on a few occasions (Nakanishi & Ototake, 1997; Olesen, 1991). In my study, fish serum samples were

collected before challenge: at 12 weeks post-vaccination (Chapter 2 and Chapter 3), nine weeks and 21 weeks post-vaccination (Chapter 4) to detect antibody levels. Serum antibodies (IgM) against *Y. ruckeri* were not found in any samples of fish serum from immersion vaccinated groups in any of the three experiments described in this thesis (Chapter 2, Chapter 3 and Chapter 4), even when the vaccinated groups appeared to have high protection with survival rates of more than 90% (Chapter 2). These results indicate that measuring the IgM antibodies in fish serum for immersion vaccine application was not enough to show success of a vaccine and that there was no correlation between antibody levels and protection. IgT plays a specialised role in gut mucosal immunity, whereas IgM contributes mainly to systemic immune responses (Zhang et al., 2010). IgT was very weakly expressed after intra-peritoneal vaccination but was highly expressed after bath vaccination with *Y. ruckeri* bacterin (Raida & Buchmann, 2007). This suggests that IgT plays a role in mucosal immunity. In my study, an anti-salmonid Ig monoclonal antibody was used in ELISA and this antibody only detected IgM in fish serum. Therefore, the additional detection of IgT levels in fish gut and gills may show the correlation between mucosal antibodies and protection. It would be useful to further investigate the measurement of IgT in the gut and gills of vaccinated Atlantic salmon.

There is a potential to use gene expression post vaccination to identify a potential biosignature as a surrogate of protection to predict vaccine success. This method would provide important advantages since it would speed up the testing process and reduce the need for animal challenges and reduce or eliminate concerns about animal welfare (Bhuju et al., 2012; Bridle et al., 2012). Methods of predicting vaccine success have been studied in producing vaccines for human, vertebrates and fish (Aranday Cortes et al., 2010; Furman et al., 2013; Jiang et al., 2014). A transcriptional biosignature of a successful immersion vaccine was identified using differentially regulated genes in the gills of Atlantic salmon after immersion vaccination against yersiniosis and before bacterial challenge (Bridle et al., 2012). The upregulation of a specific set of genes in the spleen of vaccinated Asian seabass (*Lates calcarifer*) was reported as a biomarker for the prediction of successful immune defence against *Streptococcus iniae* infection (Jiang et al., 2014). Chapter 2 investigated the expression of nine genes, out of 17 genes

reported previously (Bridle et al., 2012), as a surrogate of protection. The results confirmed that upregulation of selected genes may be used to predict the success of a *Y. ruckeri* vaccine. However, six other genes could not be shown useful as predictors of vaccine efficacy. This included four genes that were excluded from analysis due to their amplification efficiency being outside the acceptable range, while two other genes showed opposite results to those reported previously (Bridle et al., 2012) which needs to be further investigated to improve our understanding of this potential biosignature in *Y. ruckeri* vaccine.

5.2 FACTORS AFFECTING CHALLENGE EXPERIMENTS TO EVALUATE IMMERSION VACCINATION

The outcome of a challenge experiment is influenced by the challenge methods used, such as i.p. injection challenge or immersion challenge or cohabitation challenge (Chettri et al., 2015b; Nordmo & Ramstad, 1997). It was recommended that the control treatment should have the lethal rate of at least 60% and effective protection was suggested as over 60% (Amend, 1981). When using the same strain, the choice of dose for challenge is important in the evaluation of vaccine efficacy. The details of different challenge doses and the mortality of the control groups are summarised in Table 5.1. Costa et al. (2011) used the challenge dose of 4.3×10^6 CFU m^{-1} for immersion in *Y. ruckeri* for one hour on Atlantic salmon after 6 weeks post vaccination at 2g fish. The results gave more than 80% mortality of the control group. In Chapter 2, lower challenge dose of 9×10^5 CFU m^{-1} was used with the same challenge method and at 12 week post vaccination and the challenge group had 50% mortality that was lower than expected. The size of the fish used in Chapter 2 was bigger than the fish used in the study of Costa et al. (2011). They were 5 g at vaccination and were challenged 12 weeks later. In Chapter 3 and Chapter 4, the mortality rate of the control groups were higher than expected. It is recommended that the different challenge doses should be tested before the challenge using fish the same size as the experimental fish to make sure that the mortality rate in the control group is around 60%.

Table 5.1 Challenge doses of *Y. ruckeri* used for Atlantic salmon by immersion challenge for 1 h

Fish size at vaccination (g)	**Time for challenge** (weeks post vaccination)	**Challenge dose** (CFU/mL)	**Mortality of control group** (%)	**References**
2	6	4.3×10^6	> 80	Costa et al. (2011)
5	12	9.0×10^5	50	Chapter 2
2	12	2.5×10^8	78.9	Chapter 3
0.13	21	2.5×10^7	90	Chapter 4

The methods to quantify bacteria and the accuracy of the quantification may affect the challenge dose. The dose for challenge was confirmed by inoculating bacterin on TSA plates and it took 36 hours to know the results (Table 5.1). Before challenge, *Y. ruckeri* numbers were estimated using Neubauer hemocytometer after inactivating the cells with formalin. Counting error may occur due to the cells overlapping other cells or missing cells if they are not recognised by the person counting the cells. Pipetting errors can contribute to challenge dose different than desired (Brecher & Cronkite, 1950). It would be useful to reduce counting errors by staining bacteria cells with Trypan Blue to make the cell recognition easier (Meyers, 2000) or developing a standard optical density curve of *Y. ruckeri* to estimate accurately the numbers of cells and thus the challenge dose (Haig et al., 2011).

5.3 FUTURE RESEARCH

As mentioned previously, a novel method of inactivating bacteria using ammonium sulphate was successfully used to produce a vaccine (Chapter 2 and Chapter 3). Although the survival rate of the ammonium sulphate vaccinated fish was not significantly different to the formalin vaccinated fish, the ability of purifying proteins promises future improvements of the vaccine. Due to the purification process, ammonium sulphate plays important role in developing vaccines in humans (Horwitz et al., 1995;

McMaster, 2000a; b). The fish vaccine industry would benefit from research on using ammonium sulphate for inactivation. This could involve further experiments with different methods of immersion vaccine. For example, single dip and double dip immunisation could be compared or combined with double dip with hyperosmotic pretreatment of the ammonium sulphate bacterin. A comparison of vaccine storage between ammonium sulphate vaccine and formalin vaccine would be useful to understand the ability of preserve antigen of ammonium sulphate.

Hyperosmotic pretreatment was used for the first time in vaccination against *Y. ruckeri* for Atlantic salmon (Chapter 3). Although RPS was not over 60% (Amend, 1981) most likely due to the high challenge dose, the hyperosmotic pretreatment was the only group significantly different to the unvaccinated group. A previous study suggested hyperosmotic pretreatment may cause stress or damage to the fish skin or gills (Nakanishi & Ototake, 1997) but my study did not show any evidence of the damage and very few studies have shown any the disadvantage of hyperosmotic pretreatment in fish. Further experiments with lower challenge dose and the effects on fish gills and stress would enhance our understanding of hyperosmotic pretreatment in fish vaccination for Atlantic salmon.

As previously stated, identification of a biosignature as a surrogate measure of protection would predict vaccine success and reduce concerns about animal welfare in disease challenges using fish. The fish vaccine industry would also benefit from further work into the transcriptomic response of host immune organs to vaccination if it could generate biomarkers that would predict vaccine success for particular species and diseases. The expression of six genes, out of the 17 genes identified by Bridle et al. (2012), was not measured in this study. These genes can be further investigated in the ammonium sulphate vaccinated fish. The measurement of gene expression of these 17 genes in a commercial vaccine Yersinivac-B or a formalin inactivated vaccine could provide clearer evidence of this potential biosignature in prediction vaccine success on yersiniosis.

The need of early vaccination for early life stages of Atlantic salmon is important. However, the sizes when fish have fully developed immunocompetence so they are protected after vaccination need to be further investigated. The only study on the immunocompetence at different size of salmonids is a study of Johnson et al. (1982) and it is on rainbow trout size against *Y. ruckeri* vaccine and the sizes of other salmonids species against *Vibrio anguillarum*. It would be useful to understand development stages of Atlantic salmon by conducting further experiment with different time points of immersion vaccine when fish are more than 1 g size or combine immersion with oral immunisation in fish smaller than 1 g that had showed some promising results (Ghosh et al in prep., Appendix 1).

5.4 CONCLUSION

In conclusion, this thesis has increased our knowledge into different methods of immersion immunisation of Atlantic salmon against yersiniosis. The successful use of a novel vaccine preparation using ammonium sulphate to inactivate the bacteria was reported for the first time for yersiniosis vaccine. Hyperosmotic pretreatment, which to the best of my knowledge has not been previously tested in Atlantic salmon, showed improved protection against *Y. ruckeri*. The results of the gene expression study supported the use of immune response genes as a biomarker of vaccine success against *Y. ruckeri* in Atlantic salmon. The potential protective nature of antibodies and the correlation with vaccine protection still needs to be further investigated including IgT levels to have better understanding of fish mucosal immunity. Finally, the study of vaccine performance of immersion immunisation of small hatchery-sized fish has contributed to our understanding of the effects of administration strategies on vaccination for small fish.

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APPENDIX 1: Associated Research Publication

Comparative protection achieved by mucosal immunisation of first-feeding Atlantic salmon, *Salmo salar* L., against yersiniosis by oral and immersion routes

B. Ghosh, T. D. Nguyen, P. B. B. Crosbie, B. F. Nowak, A. R. Bridle

Institute for Marine and Antarctic Sciences, University of Tasmania, Locked Bag 1370, Launceston, Tasmania 7250, Australia

(Submitted for peer review)

Highlights

· Significant protection from microencapsulated oral vaccine

· Prolonged protection in absence of adaptive immune response

· No effect of treatments on asymptomatic infection rates

Abstract

Yersinia ruckeri is a ubiquitous pathogen of finfish capable of causing major mortalities within farmed fish stocks. It can be transmitted vertically from parent to progeny as well as horizontally in the water column from both clinically infected fish and asymptomatic carriers, and therefore capable of infecting fish at early stages of development. Immunisation strategies that can protect small fry are therefore critical for the effective management of fish health, as is the ability to detect fish carrying covert infection. In this study, first-feeding Atlantic salmon fry were immunised either by oral administration of a microencapsulated *Y. ruckeri* vaccine formulation, or via immersion in bacterin suspension, with and without a booster immersion vaccination at 1 g size. Protection in groups receiving only immersion immunisation did not differ significantly from untreated controls when challenged with *Y. ruckeri* at approximately 5 g size, while orally immunised fish were significantly better protected than untreated controls ($F = 4.38$, $df = 4,10$, $P = 0.026$), with RPS varying between 29.4% (ORAL) and 51% (ORAL+DIP). A quantitative real-time PCR assay was used to successfully detect asymptomatically infected fish among challenge survivors, indicating more than 50% of surviving fish in each group were infected with no significant differences between immunised fish and untreated controls.

Introduction

Yersinia ruckeri, a Gram-negative member of the family Enterobacteriaceae, is the causative agent of Enteric Redmouth disease (ERM) and yersiniosis in salmonids. Though first identified from rainbow trout (*Oncorhynchus mykiss Walbaum)* in the Hagerman Valley, USA [1], *Y. ruckeri* is known to cause disease in several farmed and wild species including other salmonids such as Atlantic salmon (Salmo salar L.) [2-5]. It is now a ubiquitous pathogen that has been isolated from fish populations around the world, as well as from other taxa and environmental samples [6, 7].

Y. ruckeri is capable of causing mass mortalities and significantly impacts the global salmonid culture industry. It has been reported to cause disease in Atlantic salmon stocks, and has been associated with mortalities in the Australian Atlantic salmon industry [8-10]. *Y. ruckeri* outbreaks within the Australian Atlantic salmon industry typically result in a less florid form of yersiniosis than in the northern hemisphere,

lacking the subcutaneous haemorrhaging in and around the mouth and throat that has led to the disease being described as ERM [11, 12]. Yersiniosis in Atlantic salmon grown in Australia can be detected through unilateral or bilateral exophthalmia accompanied by ocular haemorrhaging, and a distended vent and haemorrhaging at the base of pelvic and pectoral fins in advanced stages of infection [11].

Early investigations demonstrated the efficacy of immersion vaccination of fish in a bacterin as a successful strategy for protecting farmed salmonids against *Y. ruckeri*, and an immersion vaccine consisting of formalin-inactivated whole cells was commercially licensed in 1976 in the USA [13]. A similar vaccine, developed by DPIPWE Launceston, Tasmania, has been used extensively to vaccinate fingerlings (body weight 5 g) in the Australian salmonid industry. However, outbreaks still occur, and mortality of approximately 500,000 fish occurred over a six-month period in 2007 despite stocks having been vaccinated [9].

At present, salmonid fry are initially immunised against *Y. ruckeri* by immersion vaccination at approximately 2 – 5 g size [14, 15], at which point they are still too small for intraperitoneal vaccination but large enough to handle without major deleterious impacts. Before reaching this size, S. salar fry are not considered to have developed sufficient adaptive immunocompetence, and are typically not provided with any form of immunoprophylaxis. Given the ubiquity of *Y. ruckeri*, and its ability to survive in the environment without a host [7, 16], the risk of infection in small fish is considerable. Immune system components generally develop early in freshwater fish [17], and recently, bacterial challenge of rainbow trout larvae and fry has shown that first-feeding salmonids may possess a range of innate immune factors that offer protection from infection, though the mechanisms involved are not clear [18]. These findings suggest that enhancing the immune response in Atlantic salmon fry against pathogenic infection may be possible. Recently, onset of *Y. ruckeri* infection has been observed in fish smaller than the minimum vaccinated size in commercial Atlantic salmon populations, indicating the importance of developing an effective means of protecting smaller fish that is also practicable on a commercial scale.

Y. ruckeri is capable of establishing and maintaining subclinical infection, resulting in asymptomatic carriers. When stressed, these carriers instigate horizontal transfer of the

pathogen, subsequently producing clinical infection within a population [19]. Vaccination using current methods has been unsuccessful in preventing the establishment of asymptomatic carriers within stock populations, and clinical expression from pre-existing subclinical infection has been reported in various salmonid species including Tasmanian Atlantic salmon [12, 19, 20]. An immunoprophylaxis strategy capable of inhibiting establishment of asymptomatic carriers would therefore prove extremely beneficial for salmon health management.

Mucosal administration of antigens offers the most feasible approach for immunisation of small fish. Mucosal immunisation also specifically targets stimulation of mucosal immunity in the fish, which arguably provides the first line of defence against most pathogens that fish are exposed to [21, 22]. Oral immunoprophylaxis in particular represents an ideal strategy for this purpose as it has no fish-size limitations and requires minimal infrastructure and specialized skills for effective implementation. However, protection conferred by oral immunisation has proved inconsistent in trials [23-27]. Digestive degradation has been implicated as the major cause of this inconsistency, as antigenic integrity must be retained until the immunogen reaches the distal intestine, which has been identified as an immunologically active part of the gastrointestinal tract involved with uptake of antigens [28-30]. Some studies in different teleost species have also reported observations of oral tolerance, a phenomenon characterized by a decrease in immune response linked to the extended administration of orally delivered antigens in various fish species including salmonids [31-33], suggesting interrupted administration regimes to address the issue.

Biopolymeric microencapsulation of orally administered antigens has demonstrated some success in protecting fish from pathogens. Besides effectively protecting immunogenic material from digestive degradation, microencapsulation increases its bioavailability due to particulate dispersion and the potential to affect controlled release of the antigenic substance. Several biopolymers used in antigen microencapsulation are also known to have intrinsic adjuvant properties, making their use advantageous in immunoprophylaxis [34, 35].

The aim of this study was to assess comparative protection afforded to first feeding Atlantic salmon fry against bacterial challenge with pathogenic *Y. ruckeri* when immun-

ised by oral administration of a microencapsulated *Y. ruckeri* vaccine or by immersion in concentrated bacterin.

Materials and Methods

Ethics statement

All procedures on fish were performed in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes and approved animal handling guidelines (University of Tasmania Animal Ethics Committee approval Ref: A12285).

Fish source, maintenance and experimental design

Atlantic salmon (*Salmo salar* L.) were used throughout this study. For *in vivo* uptake investigation, 20 fry with a mean weight of 1 g were obtained from a commercial Tasmanian hatchery and held in a 20 L mesh enclosure within a 1000 L tank supplied by recirculating freshwater at 11°C. For immunisation studies, pathogen-free alevin were obtained from a commercial Tasmanian hatchery prior to commencement of exogenous feeding. They were divided into two groups corresponding to the two routes of immunisation – oral and immersion – and acclimated for three weeks in a UV-sterilised freshwater recirculating system at 11°C. Post-acclimation, fish at a mean weight of 0.32 g were transferred to 200 L tanks, supplied by UV-sterilized recirculating freshwater at 11°C. Other water quality parameters including pH (7.2–7.6), ammonia (<0.25 mg L^{-1}), nitrite (<0.25 mg L^{-1}), nitrate (<0.5 mg L^{-1}) were monitored daily. Exogenous feeding commenced 24 h after transfer, and fish were then fed daily to satiation with a commercial crumble feed (Skretting, Cambridge, Tasmania). Tanks were randomly allocated to specific treatment groups, comprising three replicate tanks containing 35 fish each (Table 1). Fish were starved for 24 h seven days after initial feeding, following which experimental treatment administration was commenced.

Yersinia ruckeri culture

All *Yersinia ruckeri* cultures were grown in tryptone soy media (Oxoid, Basingstoke UK), either as broth (TSB) or agar (TSA), using aseptic techniques. For immunoprophylactic preparation, 15 mL TSB was inoculated with frozen *Y. ruckeri* stock (strain UTYR001) and incubated at 18°C overnight to obtain a starter culture. Starter

culture was used to inoculate 50 mL TSB (1:100 v/v), which was incubated at 18°C overnight, and in turn used to inoculate 5 L TSB (1:100 v/v), and incubated at 18°C for 24 h with constant aeration. For bacterial challenge, the same procedure was followed to achieve a final culture volume of 7 L.

Preparation of oral vaccine

Cultured *Y. ruckeri* cells were inactivated by the addition of neutral-buffered formalin at 0.3% of total volume and subjecting the culture to constant agitation over 24 h. TSA plates were inoculated with 100 µL of inactivated bacteria in triplicate, and incubated at 18°C for 24 h to confirm bacterial inactivation. Inactivated bacteria were concentrated by centrifuging at 8000x*g* for 30 min and washed twice in PBS. Concentrated cells were combined with distilled water (4:1 v/v) and resuspended by 60 s vigorous agitation. Cells in suspension were disrupted by four cycles of ultrasonication (60 s on / 30 s off) while held on ice.

Y. ruckeri lysate was microencapsulated using methods adapted from Zheng *et al.* [36]. Briefly, lysate was combined with a 4% (w/w) aqueous solution of sodium alginate salt (medium viscosity; Sigma-Aldrich) in 1:3 ratio (v/v) and stirred for 10 min to produce a 3% final alginate concentration. This was gradually introduced into the oil phase (octane containing 7.5% v/v Span-80; Sigma-Aldrich) in a 1:2 ratio (v/v) and emulsified by stirring at approximately 8000 rpm with the addition of Tween-80 (3.3% v/v; Sigma-Aldrich). Microcapsules were calcium-crosslinked over 45 min, hardened in isopropyl alcohol for 25 min and recovered by centrifuging the mixture at 2000x*g* for 10 min. Recovered microcapsules were washed twice in distilled water and then lyophilised.

Commercial feed was lyophilised until reduced in weight by 50% and crushed to a fine powder. Lyophilized microcapsules were mixed with powdered feed (1:100 w/w) until a uniform mixture was achieved. Distilled water was added to the mixture (50% w/w) and combined to form a dry paste, which was extruded and dried at 18°C. Dried treated feed was crushed coarsely and sieve-separated to obtain particles commensurate to fish size over the duration of treatment administration.

Fluorescent labelling of vaccine microcapsules

All protocols involving fluorescein isothiocyanate (FITC; Sigma-Aldrich, St. Louis MO, USA) were performed under protection from light exposure. *Y. ruckeri* microcapsules were labelled for validation of uptake after oral administration by affecting the modifications to the oral vaccine manufacture process.

Briefly, sonicated *Y. ruckeri* lysate was fluorescently labelled by combining with FITC solution (2.5% w/w in 1M phosphate-buffered saline; PBS) at a 2:1 ratio (v/v) and incubated at 30°C for 4 h. FITC-labelled cell lysate was dialysed against 0.01M PBS over 24 h to remove superfluous FITC. To produce FITC-labelled alginate, FITC solution (in 1M PBS) was combined (0.15% v/v) with 4% (w/w) aqueous alginate solution previously adjusted to pH 9, and incubated for 1.5 h at 40°C with continuous stirring. FITC-labelled alginate solution was dialysed against distilled water for 24 h at 4°C to remove any uncoupled FITC. FITC-labelled lysate was combined with FITC-labelled alginate solution in 1:3 ratio (v/v) and stirred for 10 min to produce a 3% final alginate concentration, which was used as the aqueous phase for manufacture of FITC-labelled microcapsules, subsequently combined combine with feed as described.

Preparation of immersion vaccine

Vaccine for immersion immunisation was prepared using a 24 h culture of *Y. ruckeri* cells in TSB, grown with constant aeration at 18˚C. Culture was inactivated by the addition of neutral-buffered formalin at 0.3% of total volume followed by constant agitation over 24 h, and then stored at 4°C until used for immunisation. TSA plates were inoculated with 100 µL of inactivated bacteria in triplicate, and incubated at 18°C for 24 h to confirm bacterial inactivation. Inactivated culture was diluted 1:10 for immunisation, to achieve a final suspension of approximately 1.29×10^9 cells mL^{-1}.

Immunisation

Fish were divided into five treatment groups comprising an untreated control group (CONTROL), fish receiving orally administered vaccine (ORAL), fish receiving vaccine through oral administration as well as a booster immersion-vaccination (ORAL+DIP), fish immersion-immunised prior to commencement of exogenous feeding (1DIP), and fish that were immersion-immunised prior to exogenous feeding as

well as receiving a booster immersion-immunisation later (2DIP). Treatments for groups are summarised in Table 1.

Oral immunisation

Prior to commencement of oral immunisation treatments, all fish were fed untreated commercial feed *ad libitum* for 14 days after treatment-group allocation, during which time palatability of treated feed was assessed using 10 fish maintained in an isolated system. Vaccine-treated feed was administered according to a staggered regime to minimise potential development of oral tolerance (see Rombout *et al.* [37]). The ORAL and ORAL+DIP groups received treated feed, prepared as described, for seven consecutive days, followed by seven days of untreated feed. This 14-day regime was repeated thrice, achieving 21 days of treated feed administration in total. Following completion of the oral treatment regime, all fish were returned to untreated commercial feed until bacterial challenge. Fish in the CONTROL group were maintained on untreated commercial feed throughout, until bacterial challenge.

When at a minimum weight of 1 g (Mean weight 1.78 g), feed was withheld from fish in the ORAL+DIP group for 24 h, following which they were administered a booster immunisation via immersion. Fish from each replicate tank were immersed in 5 L of previously prepared vaccine suspension for 60 s under constant aeration, followed by transfer to running dechlorinated freshwater for 60 s before being returned to their respective tanks. Feeding with untreated commercial feed was resumed 24 h after booster immunisation.

Immersion immunisation

Prior to commencement of exogenous feeding, fish allocated for immersion immunisation (Mean weight 0.26 g) were immersion-vaccinated in 5 L of previously prepared vaccine suspension for 60 s under constant aeration, followed by transfer to running dechlorinated freshwater for 60 s before being returned to their respective tanks. When at a minimum weight of 1 g (Mean weight 1.2 g), feed was withheld from fish in the 2DIP group for 24 h, following which they were administered a booster immunisation via immersion as before. Feeding with untreated commercial feed was resumed 24 h after booster immunisation.

Sampling

Establishment of *Y. ruckeri*-free status

Immediately after transfer to acclimation tanks, 10 randomly selected individuals (approximately 0.32 g body weight) were lethally anaesthetised (5 ml L^{-1} Aqui-S; NZ, Lower Hutt, New Zealand). Each fish was rinsed briefly to remove traces of anaesthetic, homogenised in 1 mL PBS, and the homogenate was incubated for 24 h at 18°C on TSA plates for analysis of colonies using PCR to confirm *Y. ruckeri*-free status.

Oral uptake validation

To determine uptake of oral vaccine, 15 fry randomly selected prior to group allocation were transferred to an isolated system with identical environmental parameters and maintained on untreated commercial feed until approximately 1 g (Mean weight 1.3 g). Feed combined with FITC-labelled *Y. ruckeri* microcapsules was administered *ad libitum* twice over a 24 h period. Fry were lethally anaesthetised 48 h after final feed. Maintaining protection from light exposure, spleen, liver and kidney were removed and fixed in Davidson's (freshwater) fixative over 24 h, and then prepared for histology by ethanol-series dehydration, paraffin infiltration and embedding in paraffin blocks. The blocks were sectioned at 5 µm using a microtome (Microm HM340, Germany) and mounted on glass slides, all according to standard histological procedures. Prepared sections were observed under a compound microscope equipped with fluorescent illumination (Olympus BH2, Japan) and uptake of FITC-labelled microcapsules and contents was evaluated visually.

Immune response and challenge mortality

Immediately prior to commencement of bacterial challenge, six fish (approximately 5.0 g body weight) were randomly selected from each group and lethally anaesthetised. Spleens were fixed in 1.5 mL RNAlater over 24 h at 18°C, and then stored at -20°C until analysed for assessment of immune gene expression. Immediately prior to challenge, 10 fish weighing approximately 5 g each were anaesthetised (0.3 ml L^{-1} Aqui-S) and blood was collected from the caudal vein using a 0.3 mL syringe and transferred to microcentrifuge tubes. Blood was allowed to clot overnight at 4°C and serum was recovered for antibody titre analysis by centrifuging at 4°C for 10 min at 500 x*g*.

Throughout the challenge period, reisolation of *Y. ruckeri* was attempted from 20% of daily mortalities per tank by inoculating TSA plates with kidney samples excised from mortalities. Colonies were identified using *Y. ruckeri*-specific 16S ribosomal gene primers through PCR to confirm *Y. ruckeri* as the cause of mortality.

Y. ruckeri challenge

Six randomly selected fish from each tank were transferred to a pathogen-free system with identical environmental conditions and water supply (n=18 per group), to be maintained as challenge controls. Nine weeks (at 11°C) after administration of booster immunisation, fish from all three replicate tanks within each treatment group were challenged by a 60 min immersion in 15 L freshwater saturated with air and containing pathogenic *Y. ruckeri* (75 mL culture) at a final concentration of 2.5 x 10^7 colony forming units (CFU) mL^{-1}. Initially estimated by optical enumeration, the dose was confirmed as per previously published methods [38] using TSA plates incubated at 18°C for 36 h. Following immersion, fish were returned to their respective tanks. Tanks were monitored for mortalities, which were sampled as described, for 21 days post-challenge. Cumulative percent mortality (CPM) from each treatment was used to calculate the relative percent mortality (RPS) as RPS = (1 – (mean treatment group CPM/control group CPM)) x 100. Challenge control fish were mock-challenged by similar immersion in 15 L freshwater containing 75 mL sterile TSB before being returned to their respective enclosures.

Assessment of *Y. ruckeri* - specific antibody response

Adaptive immune response to vaccination by oral and immersion routes was evaluated by measuring *Y. ruckeri* – specific antibody titres in serum of treated fish. This was achieved through an enzyme-linked immunosorbent assay utilising *Y. ruckeri* lipopoly-saccharide antigen.

Production of *Y. ruckeri* lipopolysaccharide (LPS) antigen

Formalin-inactivated *Y. ruckeri* cells were concentrated by centrifuging at 4°C for 30 min at 8000 x*g*, resuspended in 2.2 mL of distilled water and combined with 0.4 mL 100 mM Tris-HCL (pH 8.0), 0.4 mL 0.5 M magnesium chloride and 1.0 mL of 8% Triton X-100. The mixture was heated in boiling water for 10 min, cooled and concen-

trated by centrifuging for 15 min at 15000 x*g*. The pellet was washed in 10 mM Tris-HCl (pH 8) / 10 mM magnesium chloride, followed by resuspension in 4 mL resuspension buffer (equal volumes of distilled water, 0.2 M EDTA, 8% Triton X100 and 2 M sodium chloride). The suspension was incubated at 37°C for 1 h, centrifuged for 15 min at 15000 x*g*, and the supernatant transferred to a fresh tube containing 0.6 mL 1 M magnesium chloride and mixed thoroughly. To this mixture, 1 mL 100% ethanol was added drop-wise, followed by incubation at 37°C for 1 h before centrifuging at 20°C for 5 min at 18514 x*g*. The transparent precipitate obtained was washed in 10 mM Tris-HCl (pH 8) / 10 mM magnesium chloride and used as antigen in the enzyme-linked immunosorbent assay.

Enzyme-linked immunosorbent assay (ELISA)

A monoclonal anti-salmonid Ig (H chain) antibody (CLF004HP; Cedarlane Laboratories, Canada) was used in an indirect ELISA to determine *Y. ruckeri* – specific antibody titres in serum. LPS antigen of *Y. ruckeri* was diluted in coating buffer to 10 µg mL^{-1} and used to coat wells in a 96-well flat bottomed plate (Asahi Glass Company, Japan) by adding 100 µL $well^{-1}$ and incubating overnight at 4°C. Excess coating solution was removed by washing three times in a low-salt wash buffer. To reduce non-specific binding, wells were incubated for 2 h with 250 µL 3% (w/v) non-fat dry milk, followed by three washes using a low-salt wash buffer.

Serum was diluted 1:100 in PBS and 100 µL $well^{-1}$ added in duplicate. Pooled hyperimmune serum obtained from IP-immunised fish from a previous study was diluted from 1:100 to 1:3200 in a series of doubling dilutions in PBS to establish a standard curve, and added at 100 µL $well^{-1}$. Plates were sealed and incubated for 2h at 18°C with gentle shaking, followed by five washes with a high salt wash buffer, which included a 5 min incubation at room temperature in the final wash step. Reconstituted horseradish peroxidase (HRPO) conjugated – monoclonal anti-salmon Ig (CLF004HP; Cedarlane Laboratories) was diluted 1:500, and 100 µL added to each well prior to incubation at 18°C for 1 h. Following incubation, wells were washed five times with a high salt wash buffer, which included a 5 min incubation at room temperature in the final wash step. Chromogen (G7431 TMB One Solution, Promega, USA) was added (100 µL $well^{-1}$) and plates were incubated at room temperature for 10 min before addition of a stop solution

(1 M sulphuric acid; 100 µL well^{-1}). The plate was read at 450 nm following 10 s of shaking (Tecan Thermo-Spectra Rainbow, Austria). Standards were assigned an arbitrary absorbance unit (AU) value, increasing serially two-fold from 3.125 AU for 1:3200 dilutions to 100 AU for 1:100 dilutions. A standard curve was generated and used to determine relative absorbance in serum from experimental samples.

Assessment of immune-related gene expression

Differential expression of Recombination activation gene 1 (RAG-1), membrane-bound Immunoglobulin-M (IgM_{MB}) and T cell receptor α (TCR-α) in spleens of fish from each group, sampled pre-challenge, was analysed by real-time quantitative PCR to assess differences in immune response elicited by the different immunisation treatments.

RNA extraction, DNA decontamination and cDNA synthesis

Pieces of spleen (approximately 2 mg) were rinsed in water to remove excess RNAlater. Samples were then incubated at 4°C with 100 µL RNA Extraction buffer [5 M guanidine isothiocyanate, 1% Triton X 100, 50 mM Tris (pH 7)], mixed with 100 µL isopropanol and precipitated by centrifugation at 16000x*g* for 10 min at RT. Supernatant was discarded, and the pellet was incubated for 10 min at 37°C in 195 µL Urea extraction buffer (4 M Urea, 0.2 M sodium chloride, 1 mM tri-sodium citrate, 1% SDS) supplemented with 5 µL Proteinase K (20 mg mL^{-1}; Bioline Australia) with occasional agitation until resuspension of the pellet was achieved. Protein, cellular debris, and detergent were removed by centrifugation in 7.5 M ammonium acetate at 14000×*g* for 10 min at 18°C, and nucleic acids were recovered by isopropanol precipitation of the supernatant at 16000x*g* for 10 min at room temperature and washed twice with ethanol. The RNA pellet was eluted over 10 min at 37°C in 180 µL molecular grade water supplemented with 20 µL 10X DNAse buffer. The suspension was combined with 3 µL of DNAse (Baseline-ZERO™, DNAse 1000 U@1U µL^{-1}), incubated for 60 min at 37°C and transferred to ice for 5 min. Digested DNA was precipitated by centrifugation in 7.5 M ammonium acetate at 14000×*g* for 10 min at 18°C. RNA was recovered by isopropanol precipitation of supernatant at 16000x*g* for 10 min at RT. The pellet was washed twice in ethanol and eluted in 20 µL water containing 20 mM dithiothreitol (DTT; Sigma-Aldrich). Eluted RNA concentration was fluorometrically quantified (Qubit RNA BR assay, Invitrogen), and an aliquot run on a 1% agarose -Tris-borate EDTA

(TBE) gel containing RedSafe™ Nucleic Acid Staining Solution (Intron; $6x10^{-5}$ % v/v) to verify RNA integrity.

A negative control was produced by pooling 2 µL of each extracted RNA sample across all the treatment groups, and then diluting in molecular grade water (1:4). A portion of each extracted RNA sample (≈500 ng) was reverse transcribed using a 50 µM Oligo dT18 primer mix [1 µL 10 mM dNTP, 2 µL 10X RT buffer, 0.25 µL RNAse inhibitor, and 0.25 µL reverse transcriptase (M-MuLV-RT)] in molecular grade water to a final volume of 20.5 µL. Reverse-transcribed samples were diluted in water (1:4), and 5 µL of each diluted sample was pooled and serially diluted five-fold to create five standards.

Asymptomatic carrier analysis

Following termination of challenge, challenge survivors were transferred from challenge tanks to one of three enclosures corresponding to each treatment group in a system free from *Y. ruckeri*. Feeding with commercial feed was resumed 24 h after transfer, and enclosures were observed for mortalities over four weeks. At the end of this period, all surviving fish from each group were lethally anaesthetised. Spleens were excised, fixed in 1.5 mL RNA preservation solution (4M Ammonium sulfate, 25 mM Sodium citrate, 10mM EDTA, pH 5.2) over 24 h at 18°C, and then stored at -20°C. Whole spleen from each fish was analysed for *Y. ruckeri* load using real-time qPCR with *Y. ruckeri*-specific 16S ribosomal gene primers to determine asymptomatic carrier status of fish in each group. Briefly, spleen was rinsed in water to remove excess fixative and then cut into pieces (approximately 2 mm x 2 mm) to facilitate efficient lysis. Samples were incubated at 37°C for 30 min in 495 µL Urea extraction buffer supplemented with 5 µL Proteinase K to lyse cells. The resulting suspension was cooled on ice for 5 min and protein, cellular debris, and detergent were removed by centrifugation in 7.5 M ammonium acetate at 14000×*g* for 3 min at 18°C. Nucleic acids were recovered by isopropanol precipitation at 14000x*g* for 10 min at RT. The nucleic acid pellet was washed twice with ethanol and eluted in 100 µL water containing 10 µM TRIS-HCL and 0.05% TritonX (v/v).

Real-time quantitative PCR (qPCR) analysis

All real-time qPCR analyses were conducted on a CFX Connect Real-Time PCR detection system (Bio-Rad) with efficiency and stringency of standard curves held to between 85-110% and 0.98-1.00.

Immune gene expression analysis

Primers and probe used for gene-expression analysis are presented in Table 2. Each PCR reaction consisted of 5 µL 2X MyTaq HS Mix (Bioline) containing 0.5X SYBR Green (Invitrogen), forward and reverse primers (400 nM each), and 2 µL DNA template in molecular grade water to a final volume of 10 µL. Cycling conditions consisted of an initial activation of DNA polymerase at 94°C for 2 min, followed by 40 cycles of 5 s at 95°C, 20 s at 55°C and 10 s at 72°C. A melt curve was initiated at 95°C for 60 s, followed by 60 s at 55°C. The melt curve was generated by increasing the temperature in 36 increments of 1°C every 10 s to a maximum of 90°C. mRNA expression levels were standardized against mean expression levels of two reference genes [elongation factor 1α (EF1α) and β-actin] were analysed by ANOVA using the qBase Plus software (Biogazelle, Belgium).

Detection of asymptomatic *Y. ruckeri* infection

Primers and probe used for detection of *Y. ruckeri* are presented in Table 2. Each PCR reaction consisted of 5 µL 2X MyTaq HS Mix (Bioline), forward and reverse primers (400 nM each), *Y. ruckeri* 16S ribosomal gene-specific Taqman probe (100 nM) and 2 µL DNA template in molecular grade water to a final volume of 10 µL. Cycling conditions consisted of an initial activation of DNA polymerase at 95°C for 3 min, followed by 40 cycles of 5 s at 95°C and 30 s at 60°C. Assay results were quantified by analysis of raw fluorescent unit (rfu) data using the CM3 mechanistic model included in the qPCR package (v. 1.4-0) for RStudio statistical computing software [39].

Statistical analysis

Analysis of realtime qPCR results from gene expression assays was performed using the qBase Plus software. All other statistical analyses were performed in R (statistical computing software) [39], with results considered statistically different when $P \leq 0.05$. Analysis of realtime qPCR results from the *Y. ruckeri* detection assay was performed

using the CM3 model in the 'qpcR' package [40] for R. Analysis of Variance (ANOVA), performed through the 'ez' package [41] for R, was used to compare differences between treatments as appropriate, using Levene's Test to verify homoscedasticity. Tukey's HSD post-hoc test was used to determine significantly different treatments. Survival curve analysis was performed using the Log-rank test in the 'survival' package for R [42], with a Bonferroni correction set to allow for multiple pairwise curve comparisons. Difference in percentage of asymptomatic carriers was tested for significance using Chi-square analysis.

Results

In vivo microcapsule uptake and content distribution

Distinct areas of fluorescence were observed in kidney, liver and spleen from fish in the ORAL and ORAL+DIP groups, indicating translocation of the FITC-labelled to immunologically important organs, and retention over 48 h post-administration (Fig. 1). No such fluorescence was observed in organs of fish from the CONTROL group.

Y. ruckeri challenge

Both ORAL and ORAL+DIP groups demonstrated moderate protection against *Y. ruckeri* challenge compared with untreated controls, with RPS values of 29.4% and 51% respectively. Protection was lower in the 1DIP (RPS = 20.4%) and 2DIP (RPS = 16.7%) groups, which were immunised only via immersion. There was a significant difference in CPM observed between the groups, with CPM in both the ORAL and ORAL+DIP groups being significantly lower than untreated controls ($F = 4.38$, $df = 4,10$, $P = 0.026$). CPM in the 1DIP and 2DIP groups was not significantly from each other or from the CONTROL group. In contrast, survival curve analysis indicated a significant difference in disease kinetics between the untreated controls and ORAL, ORAL+DIP and 1DIP groups ($X^2 = 26.06$, $P < 0.001$). Pairwise comparisons indicated that while the ORAL+DIP group was significantly different from the two immersion-vaccinated groups, the ORAL group was not (Fig. 2). No mortalities were observed in any of the challenge control (mock infected) fish.

Immune response assessment

Antibody titres were not significantly different between treatment groups and controls, and no significant differences in mRNA expression of RAG-1, IgM_{MB} or TCR-α were observed between any of the groups (data not shown).

Asymptomatic carrier status

None of the survivors exhibited any abnormal behaviour or gross physiological signs characteristic of yersiniosis when sampled. All groups included some asymptomatic carriers, based on analysis of survivor spleens for systemic presence of *Y. ruckeri* (Table 3). Percentage of asymptomatic carriers detected in each group ranged from 51.43% (ORAL+DIP) to 81.82% (1DIP), though there were no significant differences between groups.

Discussion

This study examined the possibility of effective immunoprophylaxis in first-feeding *S. salar* fry against the effects of a bacterial pathogen, *Y. ruckeri*. Two routes of mucosal immunisation, oral administration and immersion, were investigated with and without an additional booster immersion-immunisation. The oral immunoprophylactic treatment administered to first-feeding *S. salar* fry in this study clearly conferred protection against mortality due to *Y. ruckeri* infection, as evidenced by significantly lower CPM levels than untreated controls. In comparison, mortality in the 1DIP and 2DIP groups did not differ significantly from the CONTROL group. The lack of significant protection in the immersion-immunised groups appears to corroborate previous findings from attempts to protect fish in early stages of development from yersiniosis using an immersion-based approach [43], though the mechanisms responsible for protection in orally immunised groups were not evident in this study.

Prior research has indicated that *S. salar* do not attain complete adaptive immune maturity while small fry, rationalising the lack of effort directed at immunoprophylaxis of fish at this stage of development [43]. Challenge survival in the ORAL and ORAL+DIP groups indicated a long-lasting protective effect, evident at 11°C up to 100 days after cessation of oral treatment and 63 days after booster immunisation, that was conceivably adaptive in nature. However, no significant differences were observed

between immunised groups and control fish in antibody titres and regulation of IgM, RAG-1 and TCR-α mRNA transcripts, which were assayed to detect potential induction of a specific immune response [44-46]. This is in agreement with conclusions drawn by Zapata *et al.* [47] regarding the delay in development of functional immunocompetence in contrast to ontogeny of the immune system [48]. However, the lack of adaptive immunity apparently exhibited by the ELISA results and gene expression does not explain the outcomes achieved here using oral administration strategies.

The additional administration of a booster immersion-immunisation also appears to have contributed positively to immunoprophylactic performance of the orally administered antigen, demonstrated by the results of survival curve analysis indicating a significant difference between the ORAL and ORAL+DIP treatment groups. In contrast, the disease kinetics exhibited by the 2DIP group were not significantly different the 1DIP group, suggesting that protective efficacy of the booster vaccination is not simply an additive effect, but instead dependent on the immune status already achieved in fry at the time of administration. The difference in performance of the two booster immunised treatment groups, ORAL+DIP and 2DIP, may also reflect a difference in immune response resulting from the different routes of immunisation. Previous research on immunisation of teleost fry found that lower protection was achieved through early primary vaccination by immersion followed by a booster compared to primary vaccination at a later stage without a booster [49]. The investigators inferred that this was caused by immunological tolerance produced by primary immersion immunisation attempted while fry were still incapable of generating a specific immune response. They also indicated that low immune response in fry receiving primary immunisation at an early stage of development may have been a result of agglutination by congenitally derived non-specific antibodies. In the current study, similar mechanisms may have been responsible for the low survival observed in the 1DIP and 2DIP groups.

The increased survival in orally immunised groups in this study cannot be explained by a typical adaptive immune response. Conventional understanding of the innate immune response, in teleosts and in other vertebrates, has involved a naïve response to discrete pathogenic encounters, facilitated by germ-line encoded recognition of conserved molecular patterns. However, recent studies in mammalian models have demonstrated

adaptive responses in cells of the innate immune system [50], specifically in or non-specific cytotoxic cells (NCC) [51-53]. The existence of similar processes in teleosts was recently validated using Rag-1 deficient mutant zebrafish (*Danio rerio*), which exhibited adaptive immune responses to challenge with a bacterial pathogen after an initial low dose exposure to it in spite of TCR and Ig transcript expression being absent [54]. However, the underlying mechanisms have not been explained in either the mammalian or the teleost model. Immunostimulation of NCCs using orally administered adjuvants, including naturally occurring biopolymers has been successfully demonstrated in murine models [55, 56]. In light of these findings, while not specifically assessed in this study, it is possible the protection observed in the ORAL and ORAL+DIP groups is due to NCC activity. This lack of understanding regarding the specific mechanisms responsible for the protection observed here represents an important area for further investigation.

The difference in performance of the two booster immunised treatment groups, ORAL+DIP and 2DIP, may reflect a difference in immune response resulting from the different routes of immunisation. A similar phenomenon was observed in a previous study, where Channel catfish (*Ictalurus punctatus*) fry receiving a secondary (booster) immersion immunisation produced a considerably weaker immune response than those receiving a primary immunisation at the same size and age [49]. The investigators inferred that this was caused by immunological tolerance produced by primary immersion immunisation being attempted in the booster-immunised fry while still immunologically incapable of generating a specific immune response. They also indicated that low immune response in fry receiving primary immunisation at an early stage of development may have been a result of agglutination by congenitally derived non-specific antibodies. In the current study, similar mechanisms may have been responsible for the low survival observed in the 1DIP and 2DIP groups.

Assessment of orally-administered antigen uptake in this study provided clear evidence of oral administration being a viable strategy for delivery of immunoprophylactics to teleosts. Confirmation of particulate uptake in the gut was confirmed, corroborating previous research on particulate uptake in the distal intestine [57]. Studies investigating the premise of oral antigen uptake in the teleost gut have shown evidence of antigen

translocation following enteric administration to immunologically important organs [57-59]. The results in this study support these earlier findings, and clearly validate the premise of oral immunoprophylaxis for teleosts. However, while the microencapsulating material used – alginate – is known to be a potential immunostimulant, its possible contribution to the results observed cannot be assessed independently from effects of the *Y. ruckeri* vaccine in this study. In light of previous research successfully demonstrating the immunostimulatory effects of alginate in a variety of species [60-63], and particularly at early developmental stages [64], clarifying the effects of the alginate microencapsulant used in this study independently may be of value to future oral immunoprophylaxis strategies for teleost fry.

Asymptomatic infection of salmonids with *Y. ruckeri* has previously been detected in association with intestinal mucosa [20, 65]. A number of studies have demonstrated that teleost mucosal surfaces are capable of producing localized adaptive immune responses to antigens [22, 37, 66], and the possibility of inhibiting establishment of asymptomatic *Y. ruckeri* infection through vaccine-mediated adaptive mucosal responses was assessed by comparing the proportion of carriers within survivor from each group in this study. Increased protection did not translate to increased inhibition of asymptomatic infection, with qPCR-based detection showing no significant differences between surviving populations of any treatment group. This appears to further suggest that the increased protection observed in orally immunised groups was not achieved through conventional adaptive immune responses.

In conclusion, protection of *S. salar* fry against effects of bacterial infection could be achieved via oral immunoprophylaxis more effectively than through immersion immunisation in this study. A better understanding of potential specificity of the innate immune system in teleosts is, however, critical to further development of disease management strategies for fish in early stages of development. A clearer understanding of the role played by biopolymer microencapsulants as used here would also contribute to further optimisation of such oral immunoprophylaxis strategies. However, the potential for developing orally administered immunoprophylaxis as a disease management strategy for *S. salar* fry is clearly demonstrated here.

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Tables and Figures

Table 1: Viability of C6-6 in treatments and total approximate dose per fish prior to bacterial challenge

Group Label	Immunisation	Fish/Tank
ORAL	3 x (7 d treated feed / 7 d untreated feed)	20 challenged (+ 15 for sampling)
ORAL+DIP	3 x (7 d treated feed / 7 d untreated feed) + Booster Immersion (Mean Wt. 1.78 g)	20 challenged (+ 15 for sampling)
1DIP	Immersion (prior to exogenous feeding)	20 challenged (+ 15 for sampling)
2DIP	Immersion (prior to exogenous feeding, Mean Wt. 0.26 g) + Booster Immersion (Mean Wt. 1.2 g)	20 challenged (+ 15 for sampling)
CONTROL	No treatment	20 challenged (+ 15 for sampling)

Table 2: Primers and probes used for molecular analysis

Immune gene expression analysis		
RAG-1	Forward	CCT AAC ACC TCT AGG CTT GAC
	Reverse	GCT TCC CTG TTT ACT CGC
IgM_{MB}	Forward	TCT GGG TTG CAT TGC CAC TG
	Reverse	GTA GCT TCC ACT GGT TTG GAC
TCR-α	Forward	GCC TGG CTA CAG ATT TCA GC
	Reverse	GGC AAC CTG GCT GTA GTA AGC
Y. ruckeri detection/quantification		
Forward primer [11]		AAC CCA GAT GGG ATT AGC TAG TAA
Reverse primer [11]		GTT CAG TGC TAT TAA CAC TTA ACC C
Probe (Taqman)		AGCCACACTGGAACTGAGACACGGTCC

Table 3: Percentage of challenge survivors identified as asymptomatic carriers in each group, and mean splenic bacterial load (expressed as number of *Y. ruckeri* 16S ribosomal gene copies detected)

Group	Asymptomatic carriers (%)	Median Load (ribosomal 16S gene copies)
ORAL	55.56	3.0×10^1
ORAL+DIP	51.43	1.30×10^2
1DIP	81.82	3.15×10^4
2DIP	66.67	8.72×10^4
CONTROL	59.09	3.23×10^1

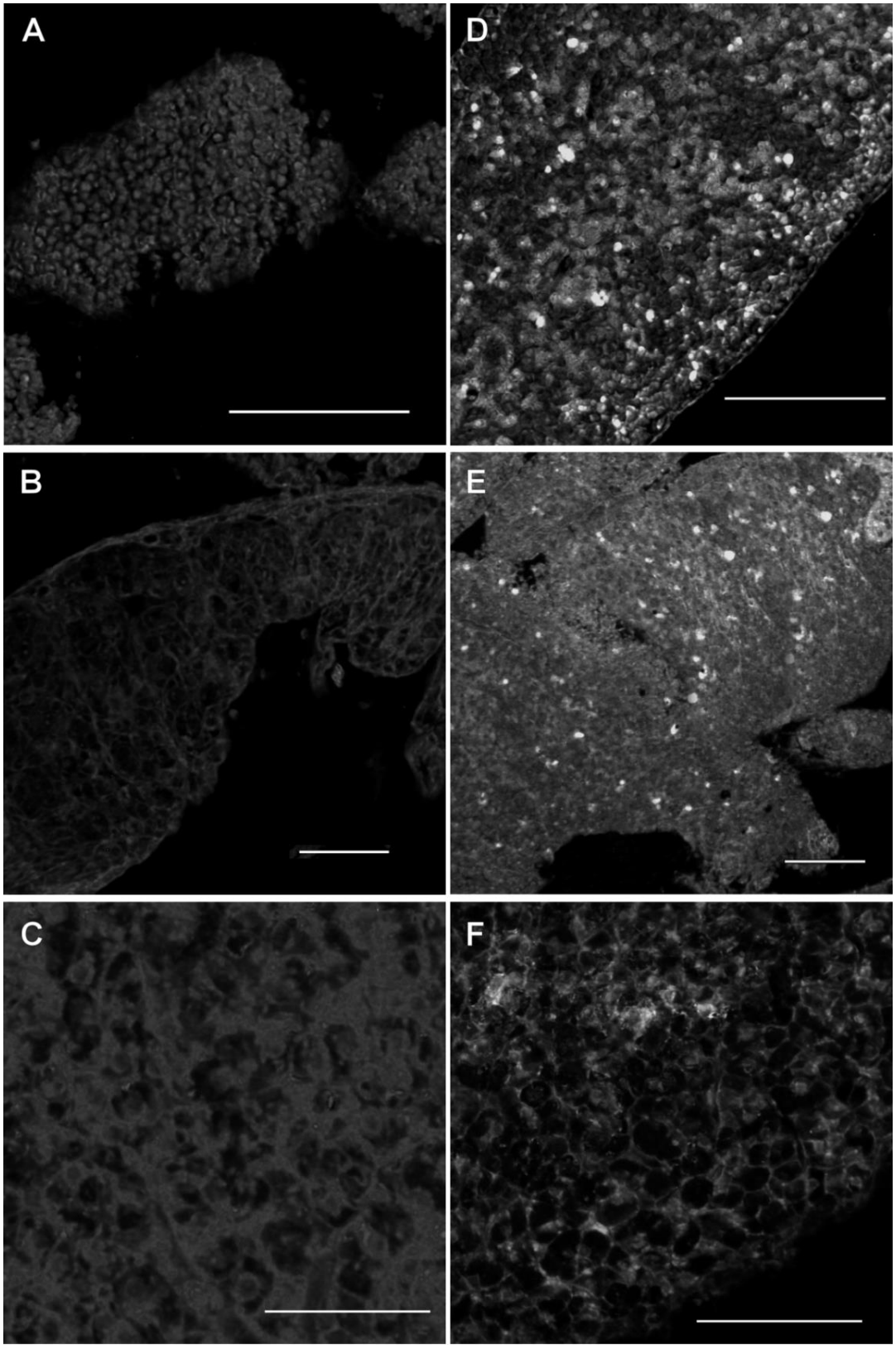

Figure 1: Fluorescent optical micrographs of samples from negative controls (A: Spleen, B: Kidney, C: Liver), and from fish fed vaccine-feed labelled with FITC (D: Spleen, E: Kidney, F: Liver). Bar=100µm

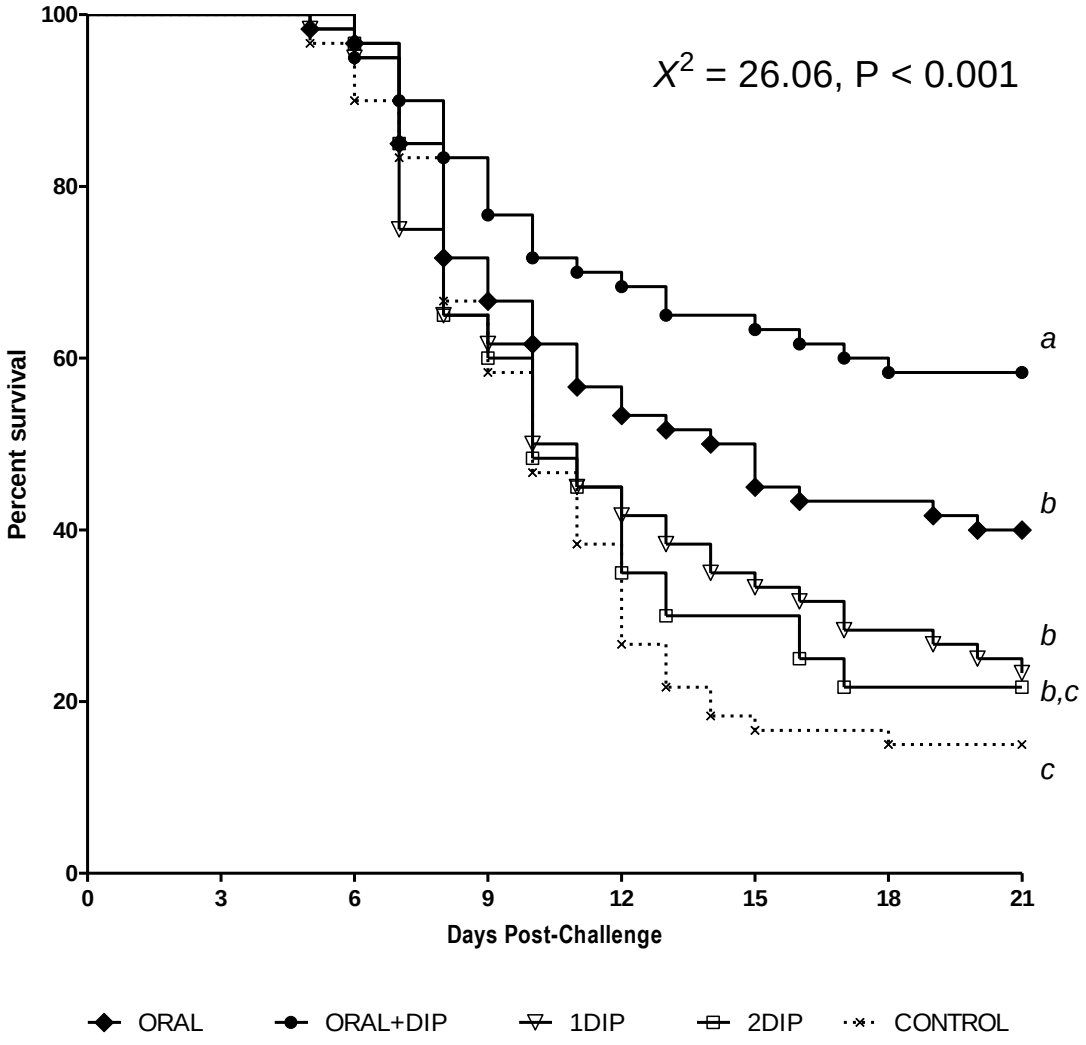

Figure 2: Survival post-challenge with *Y. ruckeri* in Atlantic salmon immunised orally or by immersion, with and without a booster immersion-immunisation at 1.0 g size. Different lowercase letters indicate significantly different treatments.